More than meets the eye – from fetal microchimerism to forensic applications

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Table of contents

List of abbreviations **11**

PART I: General introduction **13**

CHAPTER 1: More than meets the eye **15**

References 22

CHAPTER 2: Aim and outline **23**

References 26

PART II: Fetal microchimerism in autoimmune thyroid diseases **27**

CHAPTER 1: Introduction to fetal microchimerism **29**

1. Female predominance of autoimmune diseases 31
2. Fetal microchimerism 33
3. Detection of fetal microchimerism 36
   - 3.1. Detection techniques 36
   - 3.2. Fetal microchimeric cell types 39
4. Long-term consequences of fetal microchimerism: association with (non) – autoimmune diseases 42
   - 4.1. Pregnancy-associated diseases 42
   - 4.2. Non-autoimmune diseases 42
   - 4.3. Autoimmune diseases 43
     - 4.3.1. Systemic sclerosis 43
     - 4.3.2. Sjögren syndrome 44
     - 4.3.3. Systemic lupus erythematosus 44
     - 4.3.4. Primary biliary cirrhosis 44
     - 4.3.5. Autoimmune thyroid diseases 45

References 46

CHAPTER 2: Aim and outline **55**

References 58

CHAPTER 3: Introduction to autoimmune thyroid diseases **59**

1. Autoimmune thyroid diseases 61
   - 1.1. Graves' disease 61
   - 1.2. Hashimoto's thyroiditis 63

2. Susceptibility factors for AITD 65
2.1. Genetic factors 65
2.2. Non-genetic factors 66
2.3. Fetal microchimerism 67
References 68
CHAPTER 4: Male fetal microchimeric cells in blood of women with an autoimmune thyroid disease 71
Abstract 73
Introduction 74
Materials and methods 76
Ethics Statement 76
Study participants 76
Fluorescence in situ hybridization (FISH) 76
Fluorescence scanning 78
Segmentation and masking 78
Repeated FISH 79
Phenotyping of the fetal microchimeric cells 80
Statistical analysis 80
Results 81
Study participants 81
Male fetal microchimerism in patients and healthy volunteers 84
Flow cytometry for determination of purity of EasySep isolated cells 85
Fetal microchimeric cells in PBMC subsets 85
Discussion 86
Conclusion 88
References 89
CHAPTER 5: Fetal microchimerism in autoimmune thyroid diseases: harmful, beneficial or innocent for the thyroid gland? 91
Abstract 93
Autoimmune Thyroid Diseases 94
Fetal microchimerism in AITD 94
Fetal microchimeric cells in autoimmune thyroid diseases: harmful, beneficial or innocent for the thyroid gland? 96
Harmful 97
Beneficial 100

Innocent 102
Conclusion 103
References 104

PART III: Forensic applications 109
CHAPTER 1: Aim and outline 111
References 114
CHAPTER 2: Fast nuclear staining of hair roots as a screening method for successful STR analysis in forensics 115
Abstract 117
Introduction 118
Materials and methods 119
Staining of hair roots with 1 hour incubation in DAPI (part I) 119
DAPI-staining of hair roots directly on microscope slides (part II) 119
Hairs on adhesive films 119
Forensic cases 120
Microscopic evaluation of the staining 120
DNA extraction 120
DNA amplification and detection 120
Statistical analysis 120
Results and discussion 121
1 hour incubation in DAPI (part I) 121
Direct staining of hair roots with DAPI on microscope slides (part II) 121
Hairs on adhesive films 124
Success rate experimental data 124
Forensic cases 124
Conclusion 126
References 127
CHAPTER 3: Evaluation of a visualization assay for blood on forensic evidence 129
Abstract 131
Introduction 132
Materials and methods 134
Samples 134
Visualization assay 134

Kastle-Meyer test 134
LumiScene test 135
Fluorescein test 135
DNA extraction 135
DNA amplification and capillary electrophoresis 136
Results and discussion 137
Detection limit of the visualization assay 137
Combination of the visualization assay with other presumptive blood tests and subsequent DNA profiling 138
Influence of the type of fabric 139
Conclusions 141
References 142

PART IV: Summary and final discussion 145
References 154

PART V: Samenvatting en finale discussie 155
References 165

ADDENDUM: Male fetal microchimeric cells in blood and thyroid glands of women with an autoimmune thyroid disease 167
Abstract 169
References 172

Curriculum Vitae 175

Dankwoord 181

List of abbreviations

AF	Alexa Fluor
AITD(s)	Autoimmune Thyroid Disease(s)
APC	Antigen Presenting Cell
BPA	Bloodstain Pattern Analysis
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
CTLA-4	Cytotoxic T Lymphocyte-associated Antigen 4
DABCO	1,4-diazabicyclo (2,2,2) octane
DAPI	4,6'-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescence *in situ* Hybridization
G-CSF	Granulocyte-colony Stimulating Factor
GD	Graves' disease
GFP	Green Fluorescent Protein
GvHD	Graft-versus-host Disease
GvHR	Graft-versus-host Reaction
H&E	Haematoxylin and Eosin
HLA	Human Leukocyte Antigen
HT	Hashimoto's thyroiditis
HvGR	Host-versus-graft Reaction
IFN	Interferon
IL	Interleukin
KM	Kastle-Meyer
LMPC	Laser Microdissection and Pressure Catapulting
MHC	Major Histocompatibility Complex
NK	Natural Killer
PAPCs	Pregnancy-associated Progenitor Cells

PB	Peripheral Blood
PBC	Primary Biliary Cirrhosis
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEP	Polymorphic Eruptions of Pregnancy
ppm	parts per minute
PTC	Papillary Thyroid Cancer
PTPN22	Protein Tyrosine Phosphatase, Non-receptor type 22
RA	Rheumatoid Arthritis
RT	Room Temperature
SD	Standard Deviation
SLE	Systemic Lupus Erythematosus
SNP	Single Nucleotide Polymorphism
SS	Sjögren's Syndrome
SSc	Systemic Sclerosis
STR	Short Tandem Repeat
T3	Triiodothyronine
T4	Thyroxine
Tg(Ab)	(anti-) Thryoglobulin (antibodies)
TPO(Ab)	(anti-) Thyroid Peroxidase (antibodies)
TSH(R)	Thyroid Stimulating Hormone (receptor)
TSHRAb	Anti-thyroid Stimulating Hormone Receptor Antibodies
TSI	Thyroid-stimulating immunoglobulins
UV	Ultraviolet
vWF	von Willebrand Factor

PART I

General introduction

CHAPTER 1

More than meets the eye

The main goal of this PhD thesis was the search for male fetal microchimeric cells in blood of patients with an autoimmune thyroid disease (**Part II**). Through the course of this PhD thesis, optimization of protocols for forensic hair and blood analysis was also performed (**Part III**).

Objects in the range of 0.1 mm can be seen with the naked eye. However, for the visualization of smaller items, visualization aids are necessary. In many biological samples, the use of a (light or electron) microscope is essential to visualize cellular and subcellular structures. Light microscopy can be used to visualize plant or animal cells and bacteria and can resolve details 0.2 µm apart, while smaller molecules can be visualized with an electron microscope, resolving details 0.1 nm apart (**Figure 1**).

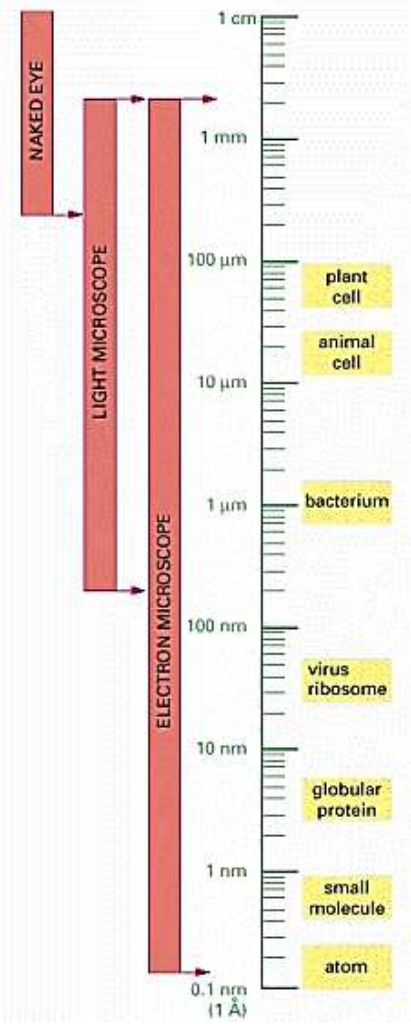

Figure 1: Resolving power. Sizes of cells and their components are drawn on a logarithmic scale, indicating the range of objects that can be seen by the naked eye and by light (and electron) microscopes [1].

Most cells however, are hardly visible through a light microscope without staining techniques. Staining techniques in this thesis were performed on non-living cells. Therefore, fixation prior to staining is necessary to preserve cellular components, prevent autolysis and displacement of cell constituents, and to stabilize cellular materials against deleterious effects of subsequent procedures. There are two types of fixations which are commonly used: cross-linking fixatives such as formaldehyde and coagulating fixatives such as ethanol [2]. In addition, if intracellular structures have to be stained, permeabilization of the cell membrane may be required. Commonly used reagents to permeabilize the cell are detergents such as Triton X-100 or NP-40.

Several specific and non-specific staining techniques have been developed. Non-specific stains generally have affinities for certain categories of molecules such as basic proteins, nucleic acids, lipids or carbohydrates [3]. Examples of non-specific, cytological stains are hematoxylin and eosin (H&E), which are commonly used in histology. Hematoxylin stains the nuclei blue whereas eosin stains cytoplasm, connective tissue and other extracellular substances pink or red. However, more specific staining methods are required to study specific proteins or other molecules in cells.

In general, fluorescent stains have a higher discriminatory power than cytological stains [4]. Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light by a fluorescent molecule and is typically of a longer wavelength than the excitation light. The difference between the excitation and emission wavelengths, known as the Stokes shift, is the critical property that makes fluorescence so powerful. Emission photons are isolated from excitation photons by appropriate filter sets. The value of fluorescence microscopy lies in the fact that, unlike other modes of optical microscopy which are based on macroscopic specimen features, fluorescence microscopy is capable of imaging with very high contrast. The use of fluorochromes has made it possible to identify specific proteins or other molecules in cells and tissues, with a high degree of specificity against non-fluorescing material. Moreover, the fluorescence microscope can reveal the presence of fluorescing material with exquisite sensitivity as molecules of interest are often present in extremely small amounts in the specimen. Several fluorescent stains can be combined to reveal simultaneously the presence of individual target molecules. The major limitation of using fluorochromes is that they lose their ability to fluoresce when they are continuously illuminated, a process called photobleaching. However, minimizing illumination or using antifade reagents such as Vectashield can overcome this problem [5].

Many fluorochromes have been developed for their use in the field of fluorescence microscopy. DAPI or 4,6'-diamidino-2-phenylindole is one of the most commonly used fluorochromes. DAPI is excited by ultraviolet light and shows strong blue fluorescence when bound to nucleic acids, preferentially to

adenosine and thymidine base pair regions [6]. DAPI can be used to detect cell nuclei in tissue sections or cell specimens (**Part II**) or can be used to visualize nuclei in hair roots (**Part III**).

Fluorescence microscopy has many clinical and forensic applications [1,4]. In immunofluorescence, a sample is treated with a primary antibody specific for the antigen of interest. A fluorochrome can be directly conjugated to the primary antibody. Alternatively, a secondary antibody conjugated to a fluorochrome which binds specifically to the first antibody, can be used. An example of a secondary antibody staining is shown in **Figure 2**.

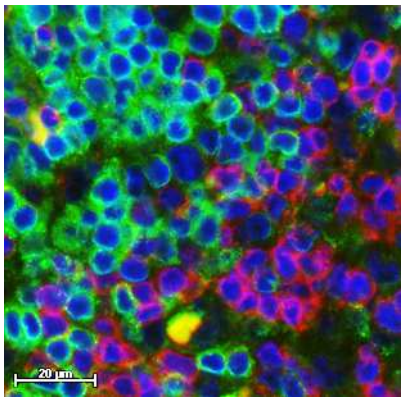

Figure 2 : In thyroid glands, T cells are stained red (mouse anti-CD3 antibody (primary) and goat anti-mouse antibody conjugated with AF594 (secondary)), while B cells are stained green (rabbit anti-CD79α antibody (primary) and goat anti-rabbit antibody conjugated with AF488 (secondary)).
Cell nuclei are stained blue with DAPI.

Fluorescence microscopy can also be used in combination with fluorescent probes to detect and localize the presence or absence of specific DNA sequences on chromosomes. This can be achieved using the fluorescent *in* situ hybridization (FISH) staining technique. Fluorescent probes bind only to those parts of the chromosome with which they show a high degree of sequence complementarity. Three main types of chromosome-specific probes are commonly used in FISH: repetitive sequence DNA probes, locus-specific DNA probes and whole chromosome painting probes. Centromeric repetitive sequence DNA probes are often applied for interphase cytogenetics as a strong fluorescent signal can be generated due to the many tandem repeated copies of the target sequence [7,8]. FISH can be used in genetic counseling (e.g. Down Syndrome), medicine (e.g. for diagnosis), or species identification (e.g. pathogen identification) [9]. FISH can also be used to detect specific RNA targets in cells, circulating tumor cells and tissue samples.

The principle of FISH is shown in **Figure 3**. Cells must first be fixed and made permeable to allow target accessibility. Subsequently, the target DNA is denatured to produce single-stranded DNA. The probe is then applied to the slide to hybridize for approximately 12 hours. Several wash steps are

performed to remove all unhybridized probes. Subsequently, DNA sequences of interest can be visualized using a fluorescence microscope [10].

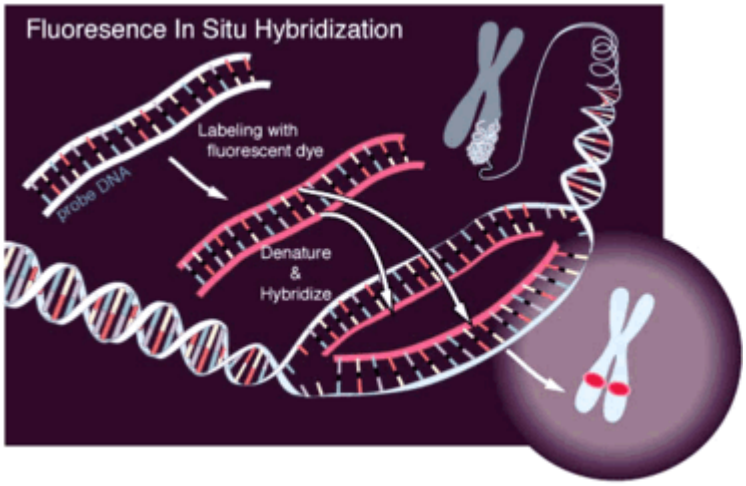

Figure 3: Principle of Fluorescent *in situ* hybridization
(http://nl.wikipedia.org/wiki/Fluorescent_in_situ_hybridization)

FISH can, for example, be used to discriminate between male and female cells by using X and Y chromosome specific probes (**Figure 4**). This can be important in the detection of male fetal microchimeric cells in female patients with an autoimmune disease (**Part II**).

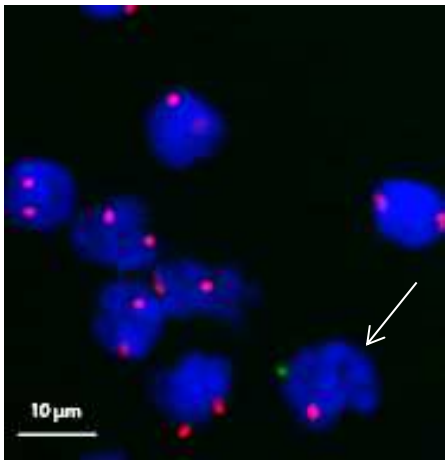

Figure 4: One male cell indicated with an arrow (one green Y-chromosomal spot and one red X-chromosomal spot), against a background of female cells (2 red X-chromosomal spots).
Cell nuclei are stained blue with DAPI.

Fluorescence microscopy can also be used for several forensic applications, e.g. for the detection of spermatozoa in a background of female epithelial cells in post-coital samples. FISH has also its applications in forensics, e.g. for the detection of male cells in a mixture with female cells in cases of sexual assault [11-13].

As described earlier, DAPI can be used to stain cell nuclei in tissue sections and cell specimens. Another application of DAPI can be found in the staining of nuclear DNA in hair roots. This can be important in forensic hair analysis to predict the DNA analysis success rate (**Part III**, **Chapter 2**) (**Figure 5**).

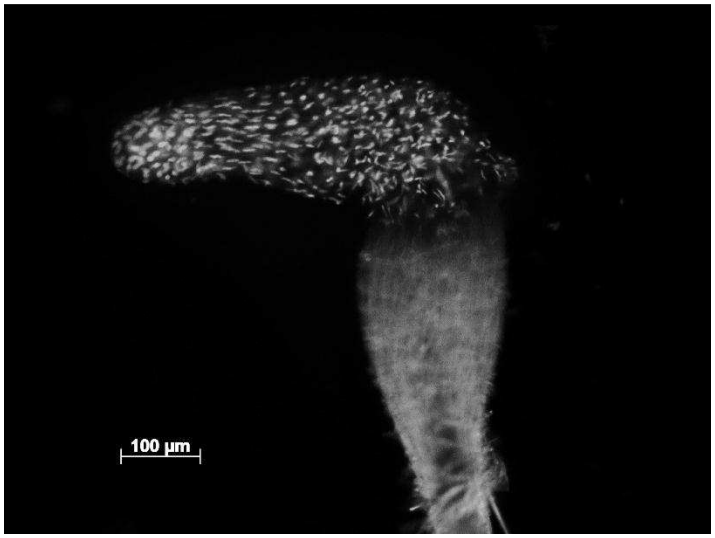

Figure 5: DAPI-staining of a hair root showing many visible nuclei.

Through the course of this PhD thesis, optimization of a visualization protocol for blood analysis was also performed. Bloodstains on dark fabrics are hard to observe with the naked eye. Therefore, a simple visualization assay using wet filter paper and pressure can be used to reveal bloodstains on dark fabrics, which is described in **Part III**, **Chapter 3**.

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CHAPTER 2

Aim and outline

The main focus of this PhD thesis was the detection of male fetal microchimeric cells in autoimmune thyroid diseases in order to investigate their potential role in the pathogenesis of these diseases (**Part II**). Autoimmune diseases show a female predominance and occur more often in the years following parturition [1]. Several hypotheses have been suggested to explain this gender difference, including differences in sex hormones, stronger immune reaction in women and X-chromosome abnormalities [2]. Another hypothesis suggests that fetal microchimeric cells, able to persist postpartum in the maternal circulation and tissues, are responsible for this gender difference [3]. An introduction to fetal microchimerism, detection methods and long-term consequences of post-partum persistence of these cells are described in depth in **Chapter 1**. In **Chapter 2**, the aim and outline of this part of the thesis are presented. Fetal microchimerism has been associated with several autoimmune and non-autoimmune diseases. In autoimmune thyroid diseases however, these cells have not been characterized. Characterizing these fetal cells would be very interesting to elucidate the potential role of fetal microchimeric cells in autoimmune thyroid diseases. In a following chapter, an introduction to autoimmune thyroid diseases is given (**Chapter 3**). In search for the potential role of fetal microchimeric cells in autoimmune thyroid diseases, blood of these patients was analyzed for the presence of male fetal microchimeric cells using FISH (**Chapter 4**). Several hypotheses concerning the potential role of fetal microchimerism in autoimmune and non-autoimmune diseases have been proposed, in which fetal cells have harmful, beneficial or innocent effects. In autoimmune thyroid diseases, only the presence of fetal microchimeric cells in blood and in the thyroid gland has been proven, but not an actual active role. In **Chapter 5**, a discussion concerning the potential harmful, beneficial or innocent role of these fetal microchimeric cells in autoimmune thyroid diseases is described. At the end of this thesis, data obtained in blood are reflected against existing data in thyroid glands (**Addendum**).

In **Part III**, a screening method for the selection of hairs suitable for forensic DNA analysis and a visualization assay for the detection of bloodstains on dark fabrics are presented (**Chapter 1**). Human hairs are frequently recovered as forensic evidence as humans shed about 150 hairs daily. The success rate of Short Tandem Repeat (STR) profiling of these hair roots is however quite low and negative results of hair analysis are frequently reported. To increase the success rate of DNA analysis of hairs in forensics, nuclei in hair roots can be visualized using the fluorescence microscope after staining the hair root with DAPI (**Chapter 2**). Bloodstains on dark fabrics are not always visible without visualization techniques. In **Chapter 3**, a visualization assay based on the application of pressure and wet filter paper is presented.

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PART II

Fetal microchimerism in autoimmune thyroid diseases

CHAPTER 1

Introduction to fetal microchimerism

1. Female predominance of autoimmune diseases

The immune system is a complex network of cellular and protein components designed to protect the individual from invasion by pathogens. Therefore, the immune system has to be able to differentiate between 'self' and 'non-self' antigens. Recognition of 'non-self' leads to the neutralization or destruction of foreign pathogens. Autoimmunity occurs when the immune system starts recognizing 'self' as 'non-self' and attacks the tissues of its own host, causing tissue damage and thereby leading to disease. In a normal state, self-reactive lymphocytes are deleted in the thymus gland (central tolerance). The few autoreactive T cells that escape central tolerance are eliminated by peripheral tolerance mechanisms [1]. If these regulatory mechanisms fail, autoimmune diseases can occur [2].

There are more than 80 different known autoimmune diseases which can range from organ-specific diseases (e.g. thyroid gland in Graves' disease) to generalized multi-system disease (e.g. systemic sclerosis). Autoimmune diseases affect up to 5% of the population [3] and are characterized by inflammation and the production of a wide range of autoantibodies directed against multiple auto-antigens. Although their etiology is still poorly understood, genetic, immunological, hormonal, and environmental factors are major predisposing and triggering factors [4].

As a group, autoimmune diseases are more common in adults than in children, and more common in females than in males [3,5,6]. The female: male ratio for some autoimmune diseases is shown in **Table 1**.

Table 1: Gender ratio (female: male) of a few autoimmune diseases [3,7-9].

Autoimmune Disease	F: M ratio
Graves' disease	3,5 – 7,2 : 1
Hashimoto's thyroiditis	5,2 – 50 : 1
Multiple sclerosis	1,8 – 4,3 : 1
Primary biliary cirrhosis	7,8 – 16 : 1
Rheumatoid arthritis	2,7 – 4 : 1
Systemic sclerosis	3,0 – 11,8 : 1
Sjörgen's syndrome	4,0 – 20 : 1
Systemic lupus erythematosus	7,4 – 10 : 1

The precise cause of this gender bias remains unknown and relatively few hypotheses have been proposed to explain why women are more prone to autoimmune diseases, including differences in sex hormones, stronger immune reaction in women and X-chromosome abnormalities. Estrogens, androgens and prolactin have been the first proposed candidates to have important roles in the sex bias observed in autoimmunity due to their capacity of modulating the immune response through androgen and estrogen receptors. Progenitor and mature cells express both receptors. These sex hormones could have a direct influence on the homing of lymphocytes to target organs and antigen presentation [4,9]. Estrogens act as enhancers of humoral activity by enhancing the production of IFNγ, IL-1 and IL-10, while androgens and progesterone act as immune suppressors by suppressing the production of IL-4 and IL-5. Prolactin stimulates both cell and humoral-based immunity [10]. However, no significant differences in hormone levels between women with an autoimmune disease and healthy control persons were observed, indicating that other gender associated differences are attributing to the high female predominance in autoimmune disorders [9,11]. Furthermore, exogenously administered sex hormones do not have similar effects on different autoimmune diseases or on susceptibility to the disease [12]. Although sex hormone levels increase during pregnancy, pregnancy has dissimilar effects on several autoimmune diseases (e.g. pregnancy commonly induces remission of rheumatoid arthritis but not of systemic lupus erythematosus) [12,13].

Women show a stronger, more vigorous humoral (antibody-mediated) immune reaction compared to men and have increased cell-mediated responses following immunization, while men produce a more intense inflammatory response to infectious organisms [11]. However, the significance of these differences remains poorly defined since there does not appear to be significant differences in susceptibility to infection or inflammation degrees between both sexes [9].

X chromosome abnormalities such as skewed X chromosome inactivation and mutations of X chromosome genes, have also been suggested as possible explanation for the female predominance of autoimmune diseases. X chromosome inactivation is an epigenetic regulation in early development that results in transcription inactivation of one of the two X chromosomes. As a result, the X chromosome inherited from either parent is silenced at random, and normal women are thus a mosaic of two cell populations. It is therefore conceivable that skewed X chromosome inactivation, when one of the two alleles (either from mother or father) is in the active X chromosome in more than 75% of cells, could lead to the escape of X-linked self-antigens from presentation in the thymus or in other peripheral sites that are involved in tolerance induction, inadequate thymic deletion, and finally, loss of T cell tolerance [10,14]. Examination of X chromosome inactivation patterns, by analyzing the methylation status of a highly polymorphic CAG repeat in the androgen receptor gene,

in peripheral blood from female patients with autoimmune diseases, such as systemic lupus erthematosus, did not reveal skewed X chromosome inactivation patterns [15]. In scleroderma however, a severely skewed X chromosome inactivation pattern was found in blood of these patients, but not in their skin lesions [14]. Skewed X-chromosome inactivation was also observed in 34% of female twins with autoimmune thyroid disease in contrast to 11% of the healthy controls [16,17]. Although skewed X chromosome inactivation alone does not explain the pathogenesis of autoimmune diseases, it can be an extra factor influencing the occurrence. Specific mutations of X chromosome genes cause immunodeficiency syndromes characterized by different degrees of severity. FoxP3, encoded by a gene localized in the short arm of the X chromosome, is essential for regulatory T cells and its deficiency or mutation leads to an early onset, highly aggressive, and often fatal multi-organ autoimmune disease [18].

However, none of the above mentioned reasons alone could explain the female predominance of autoimmune diseases. As most human autoimmune diseases develop preferentially in women of childbearing age and some autoimmune diseases, such as systemic sclerosis, show some clinical similarities with chronic graft-versus-host disease (cGvHD) after allogeneic bone marrow transplantation, another hypothesis has been suggested to explain the female predominance of autoimmune diseases: the presence of fetal microchimeric cells postpartum [12].

2. Fetal microchimerism

During pregnancy, fetal cells cross the placenta and enter into the maternal circulation (**Figure 1**) [19,20]. This fetal cell trafficking was first described by Georg Schmorl in 1893 when multi-nucleated syncytial giant cells were identified in the lungs of women who had died from preeclampsia. Schmorl speculated that these cells were of placental origin [21,22]. Seventy years later, male cells were detected in peripheral blood of parous women expecting a boy [23,24].

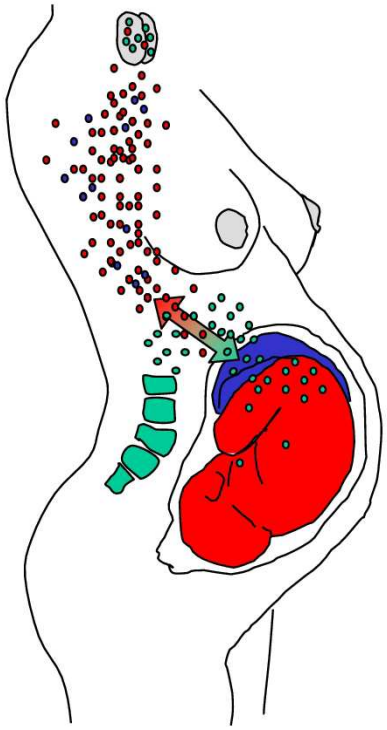

Figure 1: Cell traffic during pregnancy is bidirectional for maternal and fetal cells [25].

The term chimerism was first used by Liegeois in 1977 [26]. In Greek mythology, a chimera combines parts of different animals: a lion, a goat and a snake (**Figure 2**). In biology, the word chimerism can be used to refer to the existence of cells from genetically distinct individuals in one person.

Figure 2: chimera. (http://www.ksl.stanford.edu/software/chimaera/)

If this genetically distinct cell population is less than 1% of the total amount of cells, the term microchimerism is used. Fetal microchimerism refers to the presence of fetal cells in the mother during and after pregnancy, and is a well-known phenomenon in healthy persons as well as persons with a disease [27,28].

Fetal microchimerism is a natural consequence of normal pregnancy and results from deficiencies in the natural placental trophoblastic physical barrier tissue that separates the maternal circulation from the fetal circulation. A certain leakage is present between mother and fetus during pregnancy, and this transplacental cell trafficking is a two-way process: fetal microchimeric cells can be detected in the mother [29] and maternal microchimeric cells can be detected in the fetus/child [30].

In pregnancy, a fetal 'graft' that carries paternal and maternal antigens is by definition partially allogeneic. For a successful pregnancy, the maternal immune system must not overreact to the fetus. Several local and systemic mechanisms make sure that the fetus is not rejected by the mother, including synthesis of indoleamine 2,3-dioxygenase (IDO) and the expression of HLA-G by the syncytiotrophoblast, and a shift in Th1/Th2 balance [31]. These mechanisms indicate that pregnancy is not associated with a generalized state of immune suppression, but rather with a state of selective tolerance. Immune suppression of maternal immunity during pregnancy may play an important role in allowing the establishment of fetal microchimerism [25,32].

Fetal microchimeric cells can be detected in the maternal circulation from the 4^{th} to 6^{th} week of gestation [33-35]. Their quantity rises with gestation to peak at delivery [36]. Fetal cells can be found in the peripheral blood of almost 100% of women during the third trimester of pregnancy [37]. Quantitative studies on bi-directional transfer of cells revealed lower cell passage from mother to fetus compared to cell passage from fetus to mother [19]. The level of circulating fetal cells has been reported to be very low (2 to 6 fetal cells per ml of maternal blood during the second trimester) [38,39]. Not only do completed pregnancies result in the presence of fetal microchimeric cells in the mother, fetal microchimerism has also been described in women having a miscarriage or induced abortion. Moreover, terminations of pregnancies were associated with an elevated number of detectable fetal microchimeric cells [40-44]. In the second trimester, the number of fetal cells in the maternal circulation before and after a termination increases from about 1 to 100 per ml of maternal whole blood [40]. The number of fetal microchimeric cells in the maternal circulation is also increased by fetal and placental abnormalities, including fetal aneuploidy such as trisomy 21 [45], intrauterine growth retardation [46] and maternal preeclampsia [47,48].

Under normal circumstances, most fetal cells are lost during the postpartum period [32,33]. However, 30 to 50% of all women have detectable fetal cells in their blood [49] and tissues [29,42,50-55] decades after pregnancy, which indicates incomplete elimination of fetal cells [56]. Once fetal cells take up residence in maternal tissues [57-59] such as the thyroid gland, they may survive without being destroyed. This can be due to maternal immune adaptations during pregnancy [60], which may remain for a few months after delivery [56] allowing fetal cells to establish themselves and to survive

the postpartum period [25]. Systemic evaluation of normal organs for male cells by FISH identified male cells in thyroid, lung, lymph node and skin in women with sons and in kidney, liver and heart in women with and without sons [53]. In mice, intact fetal cells were detected in a number of maternal organs, including bone marrow, lung, heart and kidney during pregnancy [61]. Fetal cells are typically concentrated in the lungs, liver, and spleen, presumably reflecting the large blood supply of these organs [62].

The most plausible explanation described for this fetal cell persistence is the engraftment of these cells into maternal bone marrow, providing a renewing source of fetal cells in maternal blood for decades after delivery [63]. In accordance to Nelson et al. [64], we believe that human leukocyte antigen (HLA) relationship between mother and fetus is a more determining factor whether or not fetal microchimeric cells can persist in the maternal circulation and/or tissues postpartum. Increased HLA class II compatibility between mother and children was observed in women with detectable fetal microchimerism. These results led to the hypothesis that fetal cells may have crossed the placental barrier and remained unrecognized due to this HLA compatibility, establishing a low-grade, long-term state of microchimerism [65].

A recent publication stated that we are all born as microchimera [66]. Other natural sources of microchimerism besides pregnancy include cell transfusion from a twin, either a surviving twin or a vanished twin (i.e. cell transfer from a twin in utero which was lost early in gestation) and transfer of cells from an older sibling through the maternal circulation [42,44,66-71]. Iatrogenic causes of chimerism comprise blood transfusions and bone marrow or solid organ transplantations (such as kidney and heart transplantation) [72,73]. These alternative sources for microchimeric cells should be taken into account when investigating fetal microchimerism.

3. Detection of fetal microchimerism

3.1. Detection techniques

To identify male fetal microchimeric cells in women with a previous male pregnancy, male-specific gene markers can be used. Although the Y chromosome is the only universal marker of fetal microchimerism, it does not distinguish between the contributions from different male fetuses [74].

In general, two techniques have been most widely used to identify male fetal microchimerism: PCR targeting sequences specific to Y chromosome genes (e.g. *DYS14* and *SRY*) [27,75] and Fluorescence *in situ* Hybridization (FISH) using X and Y chromosome specific probes [76].

While PCR only indicates the presence of fetal cells and estimates the amount of fetal microchimeric cells [77], FISH gives an exact number [76,78]. Both techniques have a different sensitivity [79]. With

PCR, a single male cell can be detected within a background of 100.000 female cells [32,44,80] compared to 1 male cell within 2.000.000 female cells with FISH [81].

For PCR-based approaches, genomic DNA is typically extracted from blood (whole blood, plasma or peripheral blood mononuclear cells (PBMCs)) or tissues. A sequence unique to the fetus is selected for PCR amplification. In pregnancies where the gender of the fetus is male or when a woman is known to have previously given birth to a son, sequences specific to Y chromosome genes (*DYS14, SRY*) are targeted by the primers. When the fetus or child has been previously genotyped for paternally derived polymorphisms, allele specific PCR can also be used. HLA offers a broader applicable target for microchimerism detection as they are highly polymorphic and thus frequently mismatched between two individuals [82-85]. However, its sensitivity is much lower than PCR and FISH. Minimum 5 fetal cells have to be present among 100.000 maternal cells in order to be able to detect them [83,86].

Whereas the PCR-based methods of Y chromosomal detection only demonstrate the presence of male cells, other methodologies such as immunocytochemistry, FISH and HLA typing identify the location, the phenotype and the immunogenetic properties of microchimeric cells in a variety of tissues [87-89]. Moreover, the source of the microchimeric cells can be determined [49,57,90]. FISH with probes targeting X and Y chromosomes can be used to identify male cells in blood and tissues of women (**Figure 3**).

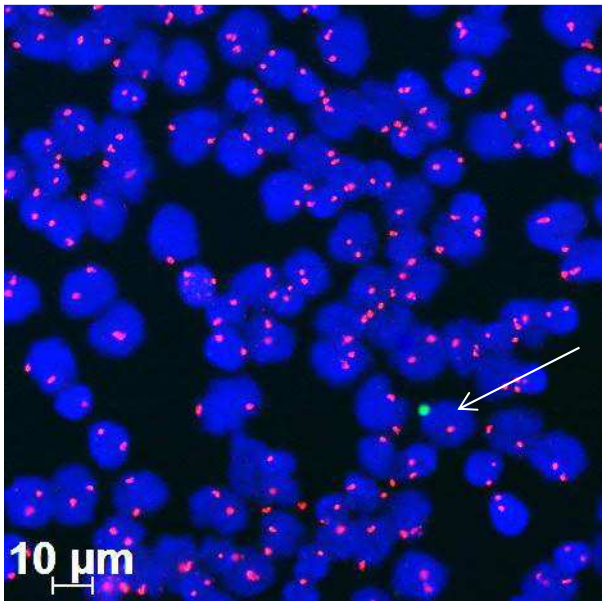

Figure 3: One male cell indicated with an arrow (one green Y-chromosomal spot and one red X-chromosomal spot), against a background of female cells (2 red X-chromosomal spots).
DAPI staining was performed to stain the cell nuclei blue.

Besides the ability to localize fetal microchimeric cells in tissue sections and the possibility to combine it with immunochemistry staining methods, FISH is less subject to contamination than PCR amplification [76]. A disadvantage of FISH is that overlapping cells can produce artifacts, implying the need for time and labor intensive experiments. Despite this disadvantage, FISH using X and Y chromosomal probes was used in our research to detect fetal microchimeric cells in patients with an autoimmune thyroid disease.

Techniques based on sex differences are currently limited by the lack of any information on female microchimeric cells, which potentially have the same consequences on maternal health as male microchimeric cells [80]. Targeting other genetic polymorphisms in tissue sections has been proposed to overcome this limitation. Focus has been put on deletion polymorphisms to develop informative FISH probes with the ability to distinguish between donor and recipient cells [91]. Alternatively, female fetal cells can be assessed by HLA-based detection but are more difficult to study because this system requires multiple detection probes [32].

FISH has been postulated to be problematic due to the frequency of false positive FISH signals and difficulties with the attribution of FISH spots to particular nuclei in tissue sections. Therefore, isolation of single candidate microchimeric cells by laser microdissection and pressure catapulting (LMPC) and low-volume on-chip multiplex PCR for DNA fingerprint analysis to identify the microchimeric cells has been proposed. Multiplex PCR using microsatellite loci allows sex-independent identification of cells and molecular genetic diagnosis by analysing specific monogenetic genomic disease markers [92].

Not only intact fetal cells have been studied, but also cell-free fetal DNA, primarily derived from apoptotic shedding of trophoblast material [19]. However, in contrast to fetal cellular microchimerism, cell-free fetal DNA and fetal erythroblasts are rapidly and definitively cleared from the maternal system postpartum [93]. Therefore, cell-free fetal DNA is less interesting to study.

In the study of fetal microchimerism, animal models can be used. In murine experiments, allogeneic male mice homozygous for green fluorescent protein (GFP) expression, were mated with wild type female mice, upon which green fluorescent fetal cells were detected in blood, bone marrow and thyroid gland of pregnant mice [94], and in maternal brain postpartum [95]. Advantages of these animal studies are the low gestational period and the opportunity to control breeding between transgenic male mice carrying the GFP gene and wild type female mice. Moreover, microchimeric cells can be well defined by immunohistochemistry or can be microdissected from the maternal tissue for further investigation [96].

There are technical issues to consider in the interpretation of results from studies of microchimerism. Because fetal microchimerism is a low-frequency event, issues of interlaboratory variability, experimental design, sensitivity and specificity of the techniques are important variables which may lead to different results and conclusions [97,98]. Variations in experimental design are demonstrated in a direct comparison between PCR of whole blood DNA and PCR of DNA from magnetically sorted cells for the detection of microchimeric cells. Magnetic cell sorting before PCR increased the sensitivity of detecting male cells by 200-fold [99]. Whole peripheral blood DNA may therefore not be useful for the reliable detection of microchimeric cells, possibly because of the high background of autologous cells [100]. Moreover, data using fresh frozen material cannot be compared to data obtained from paraffin-embedded tissue because the latter is subject to DNA fragmentation which leads to misinterpretation of the data [27]. Pathologists do not routinely change paraffin baths between samples. Another technical issue is the choice of the particular Y chromosome sequence. Some Y chromosome sequences cross-react with autosomal sequences. Multi-copy sequences have greater sensitivity than single-copy sequences (e.g. *SRY*), but some Y chromosome sequences vary in copy number from individual to individual and could give spurious results in quantitative assays (e.g. *DYZ1*). In FISH, overlapping cells in histological sections can produce artefacts. Therefore, only cells with two signals in a well-defined nucleus should be counted [101].

Furthermore, clinical variables such as pregnancy history and medication intake, should be taken into consideration [79,102]. Some reports lack pregnancy history or do not provide it in conjunction with the results of microchimerism tests. Alternative sources of microchimerism should be considered and duration of disease and therapy should be specified [101].

3.2. Fetal microchimeric cell types

During pregnancy, fetal microchimeric cells entering the maternal circulation are predominantly of hematopoietic origin such as nucleated red blood cells, T and B lymphocytes, monocytes and natural killer cells or $CD34^+$ and $CD34^+CD38^+$ hematopoietic stem cells [29,36,103,104]. Even fetal mesenchymal stem cells [52,105,106] and endothelial progenitor cells [107] have been reported. As trophoblastic cells serve as lining cells in contact with the maternal circulation, it is not surprising to find these cells in the maternal blood as a result of cell shedding [108]. The establishment of fetal $CD34^+$ or $CD34^+CD38^+$ cells in the maternal lymphoid organs or bone marrow may help to maintain tolerance to the fetal graft [29]. Fetal cells in pregnant mice express both progenitor and differentiated cell markers [109].

To persist after delivery, fetal microchimeric cells must have the ability for long-term engraftment in the maternal host and must therefore share properties with stem cells, such as unlimited self-

renewal ability and plasticity for multilineage differentiation [49,110]. These cells were termed pregnancy-associated progenitor cells (PAPCs) [57]. Their stemness is supported by the diversity of detected fetal phenotypes (**Figure 4**) and the variety of tissues into which they integrate [49,61,109]. The finding that fetal cells in maternal bone marrow harbor the potential to differentiate into different lineages, supports the idea that PAPCs might originate from one multipotent fetal stem cell type rather than being a mixture of various, less potent progenitors. However, the cellular origin of PAPCs remains elusive [74,111,112]. Whether the fetal cells actually differentiate or fuse with the damaged host cells, remains an open question. However, using chromosome-specific probes and FISH, tetraploid signals consistent with such a fusion were never observed [49,90]. In a murine model, fetal cell maturation into neurons has been demonstrated in the maternal brain, and fusion was effectively ruled out [113].

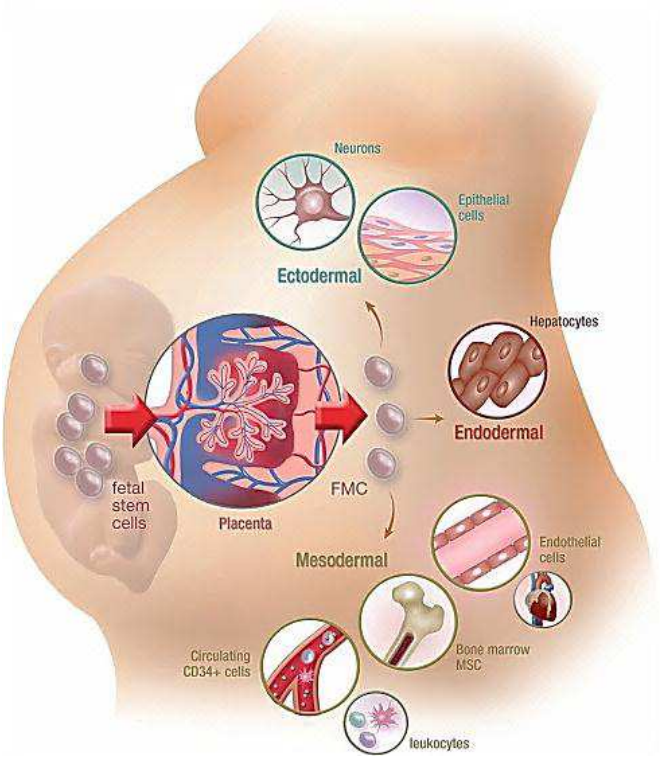

Figure 4: Fetal microchimeric cells traffic into maternal tissues and differentiate into cell types of ectodermal, endodermal, and mesodermal lineages [114].

In the postpartum period, fetal microchimerism has been described in healthy persons and persons with a disease. Combining FISH and immunochemistry, the phenotype of fetal microchimeric cells could be determined (**Table 2**).

Table 2 : Reported phenotypes of fetal microchimeric cells in human maternal tissues postpartum (adapted from [114]).

Microchimeric Cell Type	Tissue	Disease/injury	Phenotype	Reference
Epithelial	Thyroid, cervix, intestine, gallbladder	Healthy	Cyto-keratin$^+$	[57]
	Cervix	Cancer	Cyto-keratin$^+$	[115]
	Thyroid	Goiter	Differentiation into thyroid follicle	[76]
Hepatic	Liver	Healthy	Heppar-1	[57]
	Liver	Hepatitis C infection or primary biliary cirrhosis or steatosis	CAM-5.2	[90]
	Liver	Hepatitis C infection		[116]
Hematopoietic	Blood	Healthy	CD34$^+$, CD34$^+$CD38$^+$ lymphoid progenitors	[29]
	Blood	Healthy and SSc	CD3$^+$, CD19$^+$, CD14$^+$, CD56/CD16	[50]
	Lymph nodes, spleen	Healthy	CD45$^+$	[57]
	Blood	G-CSF mobilized apharesis	CD34$^+$	[28]
	Blood	Healthy	CD34$^+$	[104,117]
Mesenchymal	Bone marrow	Healthy	Vimentin$^+$	[52]
	Blood	Healthy	Vimentin$^+$	[106]
	Appendix	Appendicitis	Desmin$^+$	[118]
	Breast	Carcinoma	Vimentin$^+$	[119]
Endothelial	Intervillous space	Healthy	CD34$^+$CD31$^+$ and CD34$^+$vWF$^+$	[107]
Trophoblasts	Lungs	Preeclampsia	Multi-nucleated giant cells	[22]

4. Long-term consequences of fetal microchimerism: association with (non) – autoimmune diseases

As fetal microchimerism in peripheral blood is an almost universal finding during pregnancy [29,37,120] and postpartum [27,51], presence of fetal microchimeric cells in the circulation does not indicate an aberrant immune response by the mother. However, fetal microchimerism can persist more than 27 years [29]. Therefore, it is possible that fetal cells themselves play an active role in determining the immune repertoire. The presence of fetal microchimeric cells has been associated with later-life disease risk [12,28,29,50,120-123]. Increased fetal microchimerism is associated with some autoimmune disease [51]; while decreased fetal microchimerism is found in other malignancies [124,125]. Such associations may indicate the ability of fetal microchimeric cells of having allo-autoimmune or allosurveillance functions [48,126]. The effect of microchimeric cells on the maternal immune system and disease status remains unclear [127].

Fetal microchimeric cells have been associated with several (non-)autoimmune diseases.

4.1. Pregnancy-associated diseases

During pregnancy, microchimerism has been associated with polymorphic eruptions of pregnancy (PEP), a transient skin disease. These cutaneous eruptions occur in the third trimester of pregnancy and usually improve quite spontaneously after delivery. Male DNA was detected in 6 of 10 dermis or epidermis samples of women with PEP, whereas no fetal DNA was observed in the 26 controls [38].

4.2. Non-autoimmune diseases

The role of microchimeric cells in non-autoimmune disorders, such as hepatitis C, multinodular thyroid goiter, melanoma, cervical, lung and breast cancer is still controversial [76,115,116,119,125,128-130]. It has been speculated that the presence of these foreign cells in tissues might be the consequence rather than the cause of disease, and that they might provide a source of progenitor cells, participating in maternal tissue repair processes [115,131]. Others have suggested a role in protection by providing immunosurveillance as fetal microchimerism might contribute to reduce the risk to develop breast cancer [125,132]. However, a role in disease progression has also been considered as fetal microchimeric cells contributed to lymphangiogenesis or tumor growth in melanoma [129,132].

As breast cancer is less prevalent in parous women compared to nulliparous women, fetal microchimeric cells may reduce the risk of developing breast cancer by providing immune surveillance in blood. Fetal microchimeric DNA was less prevalent in peripheral blood of women with breast cancer than in healthy women. Fetal cells could migrate to the tumor site leading to reduced

numbers in peripheral blood [125]. According to Dubernard et al. [119], fetal cells are recruited from the peripheral blood into the damaged tissue to repair it if malignancies are developed during pregnancy. Compared to healthy controls, less fetal microchimeric cells were also observed in blood of patients with cervical cancer [54] or papillary thyroid cancer (PTC) [76,130]. These findings support the hypothesis that fetal microchimeric cells reside in maternal niches and can be recruited to the site of damaged tissues, where they could differentiate to regenerate the damaged tissue [133].

4.3. Autoimmune diseases

In 1996, Nelson postulated that some autoimmune disorders which were commonly seen in middle-aged women and had similarities with graft-versus-host disease (GvHD) occurring after hematopoietic cell transplantation, may in fact be allo-autoimmune diseases caused by fetal microchimeric cells [12]. Accumulating evidence suggests that fetal immune cells may be reactive to maternal antigens and therefore have the capacity to trigger graft-versus-host reactions (GvHR) [134]. This would provide a mechanism for the initiation and/or exacerbation of autoimmune disease.

Fetal microchimerism has been mainly described in patients with systemic sclerosis, Sjögren's syndrome, systemic lupus erythematosus and primary biliary syndrome.

4.3.1. Systemic sclerosis

The first direct discovery of fetal cells in autoimmune diseases was in patients with systemic sclerosis (SSc). SSc is an autoimmune skin disease which affects vascular and connective tissues [135]. The disease is 3 – 11.8 times more frequent in women than in men, has a peak incidence in women of post reproductive age and has striking clinical similarity to GvHD [136]. Skin, lung and esophageal involvement is prominent in both diseases, and both are characterized by lymphocytic infiltration of affected tissues, up-regulation of inflammatory cytokines and fibrosis of the dermis and visceral organs [100,136].

Fetal DNA and fetal cells were not only identified in peripheral blood and in inflammatory skin lesions of some women with SSc [12,51,134,136], but also in their salivary glands [137], spleen, lymph nodes, lung and adrenal gland [138]. Although Ohtsuka et al. [59] failed to find any difference in the frequency of women carrying male cells, the mean number of microchimeric cells was significantly higher in patients with SSc compared to healthy controls [51]. HLA compatibility was more common among patients with SSc compared to controls [51,65]. These results led the authors to hypothesize that in some cases the fetal cells may have crossed the placental barrier and remained unrecognized due to this HLA compatibility [138].

Fetal microchimeric cells expressed CD3 [136] and CD4 [55] and may react against maternal antigens [134], supporting the idea that immune responses from fetal cells may contribute to the pathogenesis of SSc [51,80,134]. Functional studies provide additional support for a role in disease pathogenesis, as CD28 stimulation of PBMCs of patients with SSc caused an increase in the amount of fetal microchimeric cells [139].

4.3.2. Sjögren syndrome

Sjögren syndrome (SS) is an autoimmune disease characterized by lymphocytic infiltration of the exocrine glands, including salivary and lacrimal glands, with systemic production of autoantibodies [137,140]. The pathogenesis of SS remains unclear. SS affects women more frequently, with a female-to-male ratio of 4-20: 1 and with the highest incidence in women older than 40 years. SS shows clinical and pathologic features resembling those of GvHD [137]. However, results of fetal microchimerism in SS are conflicting. Some studies report an increase in salivary glands of patients with SS compared to healthy controls [141,142], while other studies did not find any microchimeric cells in their glands [137,143].

4.3.3. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multi-systemic autoimmune disease characterized by autoreactive cells and autoantibodies, which can potentially affect all organ systems [144]. SLE occurs in women and men at a ratio of 7,4 – 10: 1 [145]. Investigation of fetal microchimeric cells in SLE has also yielded mixed results [142,144-146]. Patients with SLE had less frequently detectable microchimerism compared to healthy controls (0 % versus 20%) [142], while Johnson et al. [145] reported the case of a woman with SLE with high numbers of microchimeric cells in her clinically affected tissues. Microchimeric cells were detected in the maternal circulation [147,148], and in several tissues including intestines and lungs [145]. Renal biopsies of female patients with nephritis had significantly more male cells than controls [144].

4.3.4. Primary biliary cirrhosis

Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology leading to progressive destruction of small intrahepatic bile ducts by infiltrating immune cells and eventually to liver cirrhosis and failure. The disease is characterized by female predominance (7.8 - 16: 1 female: male) and is more commonly observed between the ages of 40 and 60 years [140,149]. It has many histological similarities with hepatic chronic GvHD [80].

The initial reports found that the majority of women with PBC had male DNA in their livers, but neither the frequency nor the quantity of male DNA differed significantly between healthy controls

and patients with other types of liver disease [149,150]. One subsequent report described more fetal microchimeric cells expressing CD45 in patients with PBC compared to controls. It was suggested that these male fetal cells functioned as antigen presenting cell (APC), triggering an autoimmune response to maternal tissues [151]. In the study of Stevens et al. [90], fetal microchimeric cells resembled hepatocytes and expressed a surface marker for hepatocytes In liver biopsies of women with PBC.

4.3.5. Autoimmune thyroid diseases

The main goal of this thesis was to investigate the role of fetal microchimeric cells in autoimmune thyroid diseases, which will be discussed in the following chapters.

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CHAPTER 2

Aim and outline

Autoimmune thyroid diseases (AITDs) are the most prevalent autoimmune disorders affecting up to 5% of the general population [1,2], with the two most common forms being Graves' disease (GD) and Hashimoto's thyroiditis (HT).

Although HT and GD are multifactorial diseases where genes and environmental factors influence the onset and development of the disease [3] (**Chapter 3**), fetal microchimeric cells have been proposed to play a functional role in the pathogenesis of AITD. AITD has a marked female predilection and is often detected in the years following parturition [4-6]. Fetal microchimeric cells have already been described in AITD, but the exact role of these fetal cells remains unknown [7].

The main focus of this PhD thesis was to investigate the potential role of fetal microchimeric cells in patients with HT or GD by examining their blood. In collaboration with the department of endocrinology of UZ Ghent and VUB, blood samples of patients with an AITD were obtained.

In the following chapter (**Chapter 3**), an introduction to autoimmune thyroid diseases is given. Several genetic and environmental susceptibility factors in the pathogenesis of AITD are described.

Fetal microchimerism has been investigated in blood of women with HT and GD (**Chapter 4**). By using FISH and immunocytochemistry, male fetal cells could be identified in patients and in healthy controls, and the male fetal cell type was determined.

Several hypotheses have been proposed to point out the functional role of fetal cells in several autoimmune and non-autoimmune diseases, being harmful, beneficial or innocent for the mother. In **Chapter 5**, a discussion concerning the potential harmful, beneficial or innocent role of these fetal microchimeric cells in autoimmune thyroid diseases is described.

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CHAPTER 3

Introduction to autoimmune thyroid diseases

1. Autoimmune thyroid diseases

The thyroid gland (**Figure 1**) is a major endocrine gland controlling diverse metabolic pathways by producing hormones necessary for appropriate energy levels and an active life. These hormones play critical roles in early brain development, somatic growth, bone maturation, and mRNA synthesis of more than 100 proteins that constantly regulate each and every function in the body [1,2].

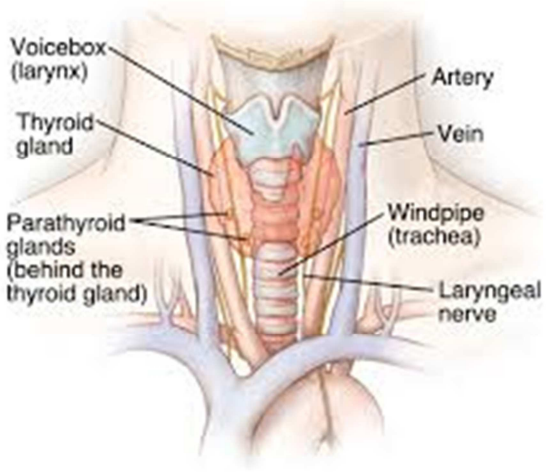

Figure 1: Position of the thyroid gland (http://www.yalemedicalgroup.org/stw/Page.asp?PageID=STW023081).

At the same time, the thyroid is highly vulnerable to autoimmune diseases due to the complexity of hormonal synthesis, tyrosine, iodine and selenium requirements for hormonal synthesis and function, and the specific capabilities of the thyroid cell to interact with the immune system by producing a variety of immunologically active factors [1,3]. Autoimmune thyroid diseases (AITDs) are therefore one of the most common autoimmune disorders. AITD occur when the immune system fails to obtain tolerance to thyroid autoantigens. The main autoantigens are thyroglobulin (Tg), which has a function in thyroid hormone storage, thyroid peroxidase (TPO), the primary enzyme involved in thyroid hormonogenesis, and the thyroid stimulating hormone receptor (TSHR). Autoimmunity to the thyroid gland results in thyroid hyperfunction or hypofunction [4]. This PhD thesis focused on Hashimoto's thyroiditis (HT) and Graves' disease (GD), the two most common forms that respectively lead to hypo- or hyperfunction [5]. Both conditions are characterized by lymphocytic infiltration of the thyroid parenchyma [6,7].

1.1. Graves' disease

Hyperthyroidism is caused by Graves' disease (GD) in 50 to 80% of the cases. The peak incidence is between 40 and 60 years of age [8]. Patients have a palpable, symmetric, smooth, and nontender

toxic goiter. In 30 to 50% of the patients, GD is associated with ophthalmopathy [8-10]. The most frequent signals of ophthalmopathy are eyelid retraction, periorbital edema, inflammation of the extraocular muscles and increase in orbital connective tissue and fat [10,11] (**Figure 2**).

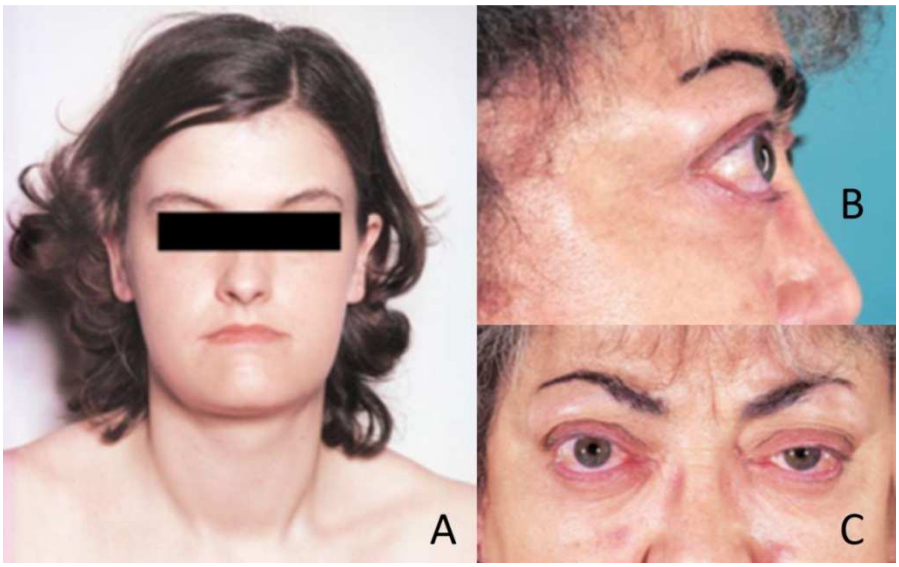

Figure 2: Clinical manifestations of Graves' disease.
Panel A shows diffuse goiter, panels B and C ophthalmopathy [10].

Symptoms of GD include nervousness, fatigue, weight loss, heat intolerance, difficulty sleeping, tremor, increased frequency of defecation, proximal-muscle weakness, irritability, and menstrual irregularity. Patients show stare, eyelid lag, tachycardia, proptosis, goiter, resting tremor, hyperreflexia, and warm, moist, and smooth skin [8,10].

GD is characterized by the presence of circulating autoantibodies that bind and activate the TSHR, stimulating follicular hypertrophy and hyperplasia and causing thyroid enlargement. Moreover, these autoantibodies lead to increases in thyroid hormone production resulting in hyperthyroidism [11-13]. Thyroid-function testing in GD typically reveals a suppressed serum thyroid stimulating hormone (TSH) level and elevated levels of serum thyroxine (T4) and triiodothyronine (T3) [10]. These patients can also have autoantibodies to Tg and/or TPO [8].

Current treatments for Graves' hyperthyroidism consist of antithyroid drugs, radioactive iodine, and/or partial thyroidectomy [10]. Antithyroid drugs, commonly used as initial therapy, can inhibit thyroid hormone synthesis. Radioiodine therapy may be used either as initial therapy or after treatment with medication in order to induce hypothyroidism to prevent a recurrence of GD. Subtotal thyroidectomy is preferred for patients with Graves' hyperthyroidism, especially those with a large goiter. It can be effective in patients with complications of antithyroid drugs, pregnant

women requiring high doses of antithyroid drugs, patients who decline treatment with radioiodine or who have large goiters or suspicious nodules [8,10].

The autoimmune process that leads to GD is believed to begin with the activation of thyroid-specific $CD4^+$ T cells, with subsequent recruitment of autoreactive B cells into the thyroid and production of anti-thyroid antibodies [14,15] (**Figure 3**).

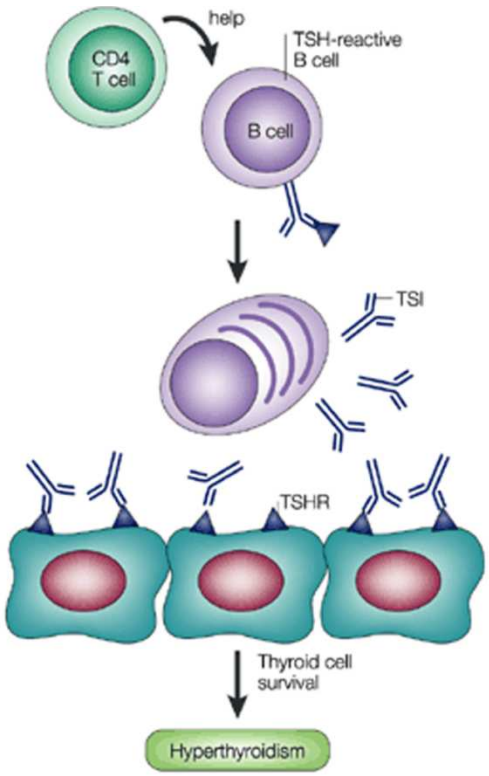

Figure 3: In Graves' disease, activated $CD4^+$ T cells induce B cells to secrete thyroid-stimulating immunoglobulins (TSI) against the TSHR, resulting in increased thyroid hormone production and hyperthyroidism [14].

1.2. Hashimoto's thyroiditis

Hashimoto's thyroiditis (HT) is the most common cause of hypothyroidism and occurs in 5 to 15% of the population [16]. It occurs most frequently in women aged between 30 and 50 years, with women comprising nearly 95% of all cases [17-19]. Patients with hypothyroidism may have a goiter (HT) or have an atrophic thyroid. Patients with HT can also present with euthyroidism [11,17].

A goiter usually develops gradually and may be found during routine examination or ultrasonography (**Figure 4**). In rare cases, the thyroid gland enlarges rapidly and the adjacent structures in the neck such as the trachea, esophagus, and recurrent laryngeal nerves, may be compressed. However, these

findings might also raise suspicion of thyroid lymphoma or carcinoma, particularly in elder patients [17].

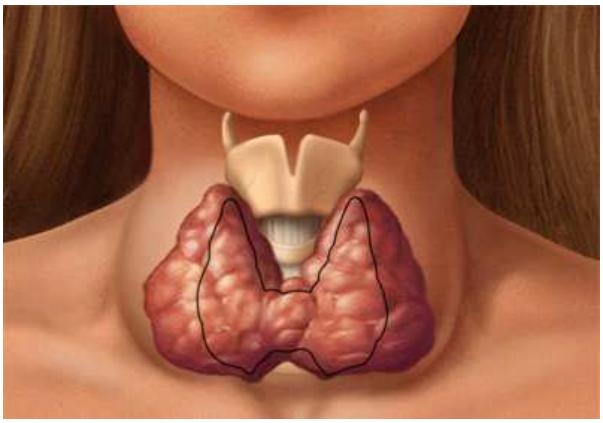

Figure 4: Enlargement of the thyroid can expand the gland well beyond its normal size (outline) and cause a noticeable bulge in the neck (http://www.mayoclinic.org/enlarged-thyroid/IMG-20005923).

HT results in impaired thyroid hormone production and clinical hypothyroidism, resulting in reduced metabolic activity in various cells and tissues. Most occurring symptoms associated with HT include weight gain, depression, difficulty in concentrating, cold intolerance, constipation, chronic fatigue, muscle weakness, bradycardia, migraines, memory loss, infertility and hair loss [12].

HT is characterized by diffuse lymphocytic infiltration, thyroid follicles of reduced size containing sparse colloid, and fibrosis replacing the thyroid parenchyma [12]. Specific autoantibodies in serum, including anti-TPO antibodies and anti-Tg antibodies are present. Thyroid ultrasonograms are useful for measuring thyroid size and assessing thyroid echo texture. Hypo-echogenicity of the thyroid may suggest severe follicular degeneration [17].

Initiation of the autoimmune reaction in HT by autoreactive $CD4^+$ T cells results in a massive lymphocyte accumulation in the thyroid gland. Activated $CD8^+$ and $CD4^+$ T cells, B cells, plasma cells and macrophages constitute the immunocyte infiltrate. Thyroid autoantibodies have been proposed to participate in hypothyroidism by blocking the TSHR. Activated $CD8^+$ T cells could be responsible for thyrocyte destruction [14] (**Figure 5**).

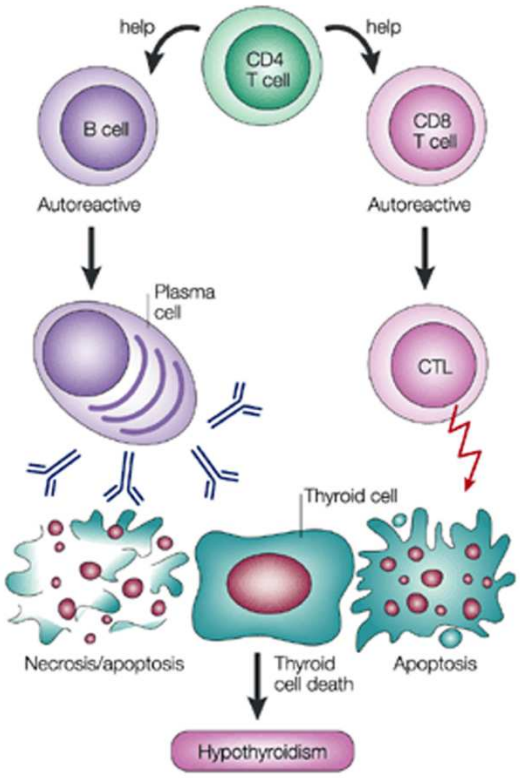

Figure 5: During Hashimoto's thyroiditis, self-reactive $CD4^+$ T cells recruit B cells and $CD8^+$ T cells into the thyroid. Disease progression leads to the death of thyroid cells and hypothyroidism. Both autoantibodies and thyroid-specific cytotoxic T lymphocytes (CTL) have been proposed to be responsible for autoimmune thyrocyte depletion [14].

2. Susceptibility factors for AITD

The etiology of AITD and the mechanisms leading to either HT or GD are unknown. However, AITD are complex diseases, caused by an interaction between susceptibility genes and non-genetic factors [7,20].

2.1. Genetic factors

HT and GD are both characterized by lymphocytic infiltrates reactive against thyroid antigens and the production of thyroid-specific autoantibodies. Genes specific to either HT or GD could explain the different pathways that the two diseases ultimately follow: the hallmark of GD is the production of TSHR stimulating antibodies causing hyperthyroidism, whereas HT is characterized by thyrocyte apoptosis, leading to glandular destruction and ultimately clinical hypothyroidism [21].

The importance of genetic factors in AITD susceptibility is supported by familial clustering, variable prevalence in different ethnic groups, associations with HLA haplotypes or single nucleotide

polymorphisms, and concordance rates in monozygotic and dizygotic twins [11,22]. In addition, population-based case-control studies, linkage analysis, and genome screens have identified several potential susceptibility genes for AITD [23,24].

The major identified AITD susceptibility genes are the HLA genes. HLA DRB1*03, DQA1*0501 and to a lesser extent DQB1*02, together making up the DR3 susceptibility haplotype, are strongly associated with GD [25-27] while HLA-DR5 is associated with HT [12,28]. DRB1*07 was negatively associated with GD, and was suggested to be protective [29,30]. Other susceptibility genes are immune regulatory genes such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), CD40 and protein tyrosine phosphatase, non-receptor type 22 (PTPN22) [7,21,24,28,31]. Obvious candidate genes for AITD are the thyroid-specific genes such as the *Tg* gene and *TSHR* gene [7,21,24,28,31]. Sequencing of the entire Tg coding sequence revealed 4 SNPs that were significantly associated with AITD [19]. TSHR is distributed in the thyroid, lymphocytes, fibroblasts, and adipocytes. TSHR-stimulating antibodies bind to the TSHR, activate adenylate cyclase, increase the rate of thyroid hormone synthesis and secretion, induce thyroid growth, and increase vascularity. Nearly all patients with GD and some patients with HT express autoantibodies against the TSHR [11]. All associated TSHR SNPs are intronic. It remains to be determined how the intronic SNPs in the TSHR gene could predispose to GD [19].

It is possible that multiple gene polymorphisms are required to develop AITD as no strong associations of susceptibility genes with AITD have been reported. However, even in a genetically predisposed person, a triggering event is usually required for autoreactivity [20].

2.2. Non-genetic factors

While the exact etiology of thyroid autoimmunity is unknown, it is believed to develop when a combination of genetic factors and non-genetic (environmental and hormonal) events leads to breakdown of tolerance. Iodine, low selenium, bacterial or viral infection, stress, smoking and drugs such as lithium can damage the thyrocytes [28,32].

Iodine is required for the synthesis of the two thyroid hormones T4 and T3. It may alter the antigenicity of Tg, forming toxic metabolites and damaging thyrocytes. In addition, iodine may generate oxygen free radicals within the thyroid, leading to direct cellular toxicity [11]. Selenium, acting as an antioxidant by reducing free radical formation, is an essential trace element that plays an important role in thyroid hormone synthesis. Low selenium can cause enlargement of the thyroid gland and thyroid hypo-echogenicity, which is a marker for lymphocyte infiltration [32]. Drugs such as lithium which have a direct inhibiting effect on the release of thyroid hormones, can also be a

trigger for AITD. Lithium-induced increases in serum TSH concentrations might enhance autoantigen expression at the surface of thyrocytes, thereby exacerbating autoimmune responses [1,32]. Bacterial or viral infection might represent a risk factor for development of AITD. Potential mechanisms of infectious triggers include cell damage with release of autoantigens, expression of new antigens, and molecular mimicry due to sequence similarities between bacterial or viral antigens and self-antigens [11]. *Yersina enterocolitica* infection has been linked with GD through molecular mimicry [6] as it has a hormone-specific binding site for the mammalian TSH that resembles the TSHR in the human thyroid gland [1]. An immune response against a viral antigen that shares homology with the TSHR may be the inductive event that ultimately leads to TSHR autoimmunity [13]. However, the role of infection is only weakly supported [20,33]. Stressful major life events have also been linked to the precipitation of AITD, particularly GD. The pathophysiologic mechanism behind the influence of such events on the autoimmune process is unclear, but could be related to alterations in the hypothalamic-pituitary adrenal axis that occur during and after the events, resulting in overall immune suppression [11]. Smoking is a risk factor for Graves' hyperthyroidism and an even stronger risk factor for Graves' ophtalmopathy [1,6,32,34], and has been associated with HT as well. However, the mechanism behind it remains unclear [1,11,32].

2.3. Fetal microchimerism

Although HT and GD are multifactorial diseases where genes and environmental factors influence the onset and development of the disease [1,35], fetal microchimeric cells, which could become activated in the postpartum period once the maternal immune suppression is lost, have been proposed to play a functional role in the pathogenesis of HT and GD [36-43].

In the following chapters, blood of patients with an AITD was analyzed for the presence of male fetal microchimerism (**Chapter 4**) and the potential role of fetal microchimerism in AITD is discussed (**Chapter 5**).

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CHAPTER 4

Male fetal microchimeric cells in blood of women with an autoimmune thyroid disease

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Abstract

Hashimoto's thyroiditis (HT) and Graves' disease (GD), two autoimmune thyroid diseases (AITD), occur more frequently in women than in men and show an increased incidence in the years following parturition. Persisting fetal cells could play a role in the development of these diseases.

Aim of this study was to detect and characterize male fetal cells in blood of postpartum women with and without an AITD. Eleven patients with an AITD and ten healthy volunteers, all given birth to a son maximum 5 years before analysis, and three women who never had been pregnant, were included. None of them had any other disease of the thyroid which could interfere with the results obtained.

Fluorescence *in situ* hybridization (FISH) and repeated FISH were used to count the number of male fetal cells. Furthermore, the male fetal cells were further characterized.

In patients with HT, 7 to 11 male fetal cells per 1.000.000 maternal cells were detected, compared to 14 to 29 male fetal cells in patients with GD ($p = 0.0061$). In patients with HT, mainly male fetal $CD8^+$ T cells were found, while in patients with GD, male fetal B and $CD4^+$ T cells were detected. In healthy volunteers with son, 0 to 5 male fetal cells were observed, which was significantly less than the number observed in patients ($p < 0.05$). In women who never had been pregnant, no male cells were detected.

This study shows a clear association between fetal microchimeric cells and autoimmune thyroid diseases.

Introduction

Autoimmune diseases affect approximately 5-8% of the population and of all the subjects with an autoimmune disease, 78% are women [1]. Many hypotheses have been proposed to explain this gender bias: differences in cytokine and hormone production in men and women, and/or differences in the degree of immune response which tends to be more vigorous in females, resulting in a higher antibody production and cell-mediated immunity after immunization [2].

Another explanation might be found in the postpartum presence of fetal cells in the maternal circulation and tissues. During pregnancy, fetal cells cross the placenta into the maternal circulation [3,4]. The immunological interaction between maternal and fetal immune cells should at that point be minimal or negligible [5]. Fetal cells can persist in the postpartum period, which indicates insufficient elimination after delivery [6]. These cells reside in maternal blood and tissues such as the skin and the thyroid [7-11]: the mother becomes microchimeric. The persistence of fetal cells may result in the development of autoimmune diseases that affect women postpartum, such as autoimmune thyroid diseases (AITDs) [12,13]. This assumption is based on the higher incidence of these diseases in women in the decades that follow parturition [13,14].

Autoimmune thyroiditis and Graves' disease are two autoimmune thyroid diseases, affecting 5-15% of women. In patients with autoimmune thyroiditis, specific auto-antibodies in serum are present, including anti-thyroid peroxidase antibodies (TPOAb), anti-thyroglobulin antibodies (TgAb) and autoantibodies binding to the TSH receptor (TSHRAb). Patients with hypothyroidism and goiter have Hashimoto's thyroiditis (HT). A variant of HT is atrophic thyroiditis. These patients present with hypothyroidism and atrophic thyroid [15]. Patients with HT can also present with euthyroidism. Graves' disease (GD) is characterized by the presence of circulating autoantibodies that bind and activate the thyrotropine receptor (TSHRAb), stimulating follicular hypertrophy and increases in thyroid hormone production resulting in hyperthyroidism [16,17]. These patients can also have TgAb and/or TPOAb [18].

HT and GD are more prevalent in women between the ages of 30 and 50 years, with a ratio female:male of respectively 10:1 and 7:1, and are often detected in the years following parturition [17,19,20]. Therefore, our study focused on these two autoimmune thyroid diseases.

It has been hypothesized that within the thyroid, the presence of fetal cells may initiate an immune response resulting in AITD [5,19]. However, direct evidence for such an effect is lacking. To our knowledge, no studies have examined which fetal cell types are present in blood of patients with HT and GD. The aim of this study was to compare the amount of male fetal microchimeric cells in peripheral blood of women with and without an autoimmune thyroid disease and to characterize the detected male fetal cells.

Materials and methods

Ethics Statement

This study was approved by the Ethics Committee of the Ghent University (B67020095877), Belgium, and written informed consent was obtained from all participants.

Study participants

The diagnosis of Hashimoto's thyroiditis was based on the presence of thyroid antibodies (TgAb, TPOAb) and hypothyroidism. The diagnosis of Graves' disease was based on the presence of hyperthyroidism, diffuse goiter, and positive serum TSH receptor antibodies. Peripheral blood (PB) was collected from 11 patients with an AITD, who had given birth to a son maximum five years before analysis. Blood was also obtained from 10 healthy volunteers who had given birth to a son maximum five years before analysis and from 3 women who never had been pregnant nor had a transfusion or transplantation. In addition, PB was collected from two women who were pregnant during the course of the study. Peripheral blood was taken just before birth of their son, 1 week and 6 months postpartum. Extreme precautions were taken to avoid external contamination. In particular, all samples were handled by a female laboratory technician.

Fluorescence in situ hybridization (FISH)

Peripheral blood mononuclear cells (PBMCs) were isolated from the patient's EDTA blood samples by density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare, Diegem, Belgium) according to the manufacturer's instructions. From each sample, 1.000.000 PBMCs were cytospun on 4 glass slides as previously described [21]. The slides were air dried and fixed for 5 minutes in a Carnoy's fixative (3:1 methanol (Fisher scientific, Leicestershire, UK): acetic acid (Sigma-Aldrich, Bornem, Belgium)).

Male fetal cells were distinguished from maternal cells by fluorescence *in situ* hybridization (FISH) with CEP X SpectrumOrange/CEP Y SpectrumGreen DNA probes (Vysis, Abbott Molecular, Illinois, US). Male cells showed one SpectrumGreen Y FISH dot and one SpectrumOrange X-FISH dot, while female cells contained two SpectrumOrange X FISH dots (**Figure 1**).

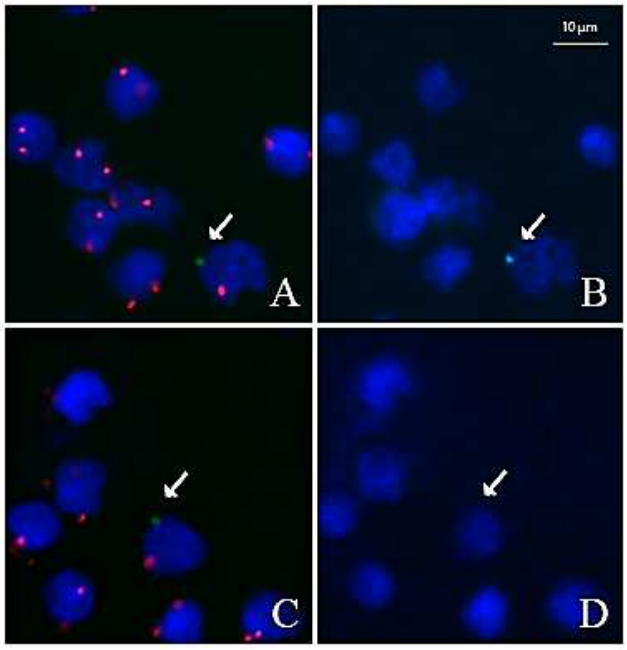

Figure 1. FISH and Repeated FISH: A. FISH of female cells, showing two SpectrumOrange X FISH spots and of a presumed male cell indicated by an arrow, showing one SpectrumOrange X FISH and one SpectrumGreen Y FISH spot; B. Repeated FISH of the female cells and the presumed male cell, showing no SpectrumAqua Y FISH spots in the female cells. In contrary, the male cell shows one SpectrumAqua Y FISH signal on the exact same location as the SpectrumGreen Y FISH spot in image A (indicated by an arrow), indicating this is a true male cell. C. FISH of female cells and of one presumed male cell, indicated by an arrow. D. Repeated FISH of a male cell (C) shows no SpectrumAqua Y FISH spot. The SpectrumGreen Y FISH spot was probably caused by cellular debris or dust particles. The SpectrumOrange X FISH spot of that cell is more diffuse than the other SpectrumOrange spots which may indicate two SpectrumOrange X FISH spots lying very closely to each other.

FISH was performed following the manufacturer's instructions with minor adjustments. Samples were incubated in a 0.01% pepsin (Serva Electrophoresis, Heidelberg, Germany)/0.01M HCl (Sigma-Aldrich)-solution during 30 min at 37°C and washed with PBS (Invitrogen, Paisley, UK) and washing buffer (1x PBS, 0.5M $MgCl_2$ (Sigma-Aldrich)). In the next step, cells were fixed for 10 min in 1% formaldehyde (Acros Organics, Geel, Belgium), rinsed with PBS, dehydrated for 3 min using an ethanol series (70%, 90% and 99%, Merck, Darmstadt, Germany) and air-dried. Afterwards, DNA was denatured by heating the slides in a denaturation solution (70% formamide (Sigma-Aldrich), 2x SSC (Vysis)) for 5 min at 73°C. Slides were dehydrated again for 1 min using an ethanol series (70%, 90% and 99%). Slides were dried on a hot plate (50°C) and 5µl of the pre-denatured probe-mixture was added on each slide. After applying a coverslip, hybridization was performed at 42°C overnight. Subsequently, slides were rinsed for 5 min with preheated 0.4x SSC/0.1% NP-40 (Sigma-Aldrich) solution at 73°C and three times for 2 min at room temperature (RT) with 2x SSC/0.1% NP-40. After air-drying, the slides were mounted with antifade Vectashield mounting solution (Vector Labs,

Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, 400 ng/ml, Sigma-Aldrich) to counterstain all nuclei on the slide. A coverslip was applied.

Fluorescence scanning

The scanning stage was controlled by the AxioVision 4.6.3 software (Carl Zeiss, München, Germany), using the MosaiX module. Image acquisition was carried out with the AxioVision multichannel fluorescence module and the AxioCam MRm camera (Carl Zeiss). Cell nuclei were visualized using Zeiss filter set no. 49 (G 365 nm, FT 395, BP 445/50), Y chromosome spots with Zeiss filter set no. 38 (BP 470/40, FT 495, BP 525/50) and X chromosome spots with filter set no. 20 (BP 546/12, FT 560, BP 575-640). Slides were scanned at 20x magnification using a Carl Zeiss short distance Plan-Apochromat® objective [21]. From every slide, 582 images were acquired and were stored as separate tiff-files.

Segmentation and masking

For automatic detection of the male fetal cells, the image processing AxioVision Commander module (Carl Zeiss) was used. All steps of processing, analysis, and evaluation were stored in an AxioVision Commander Script, which could be run automatically on the stored images. This script was based on previously published scripts with some specific modifications (**Figure 2**) [21,22]. SpectrumOrange X chromosome FISH signals were used as a visual control. The validation of the script has been described earlier [21].

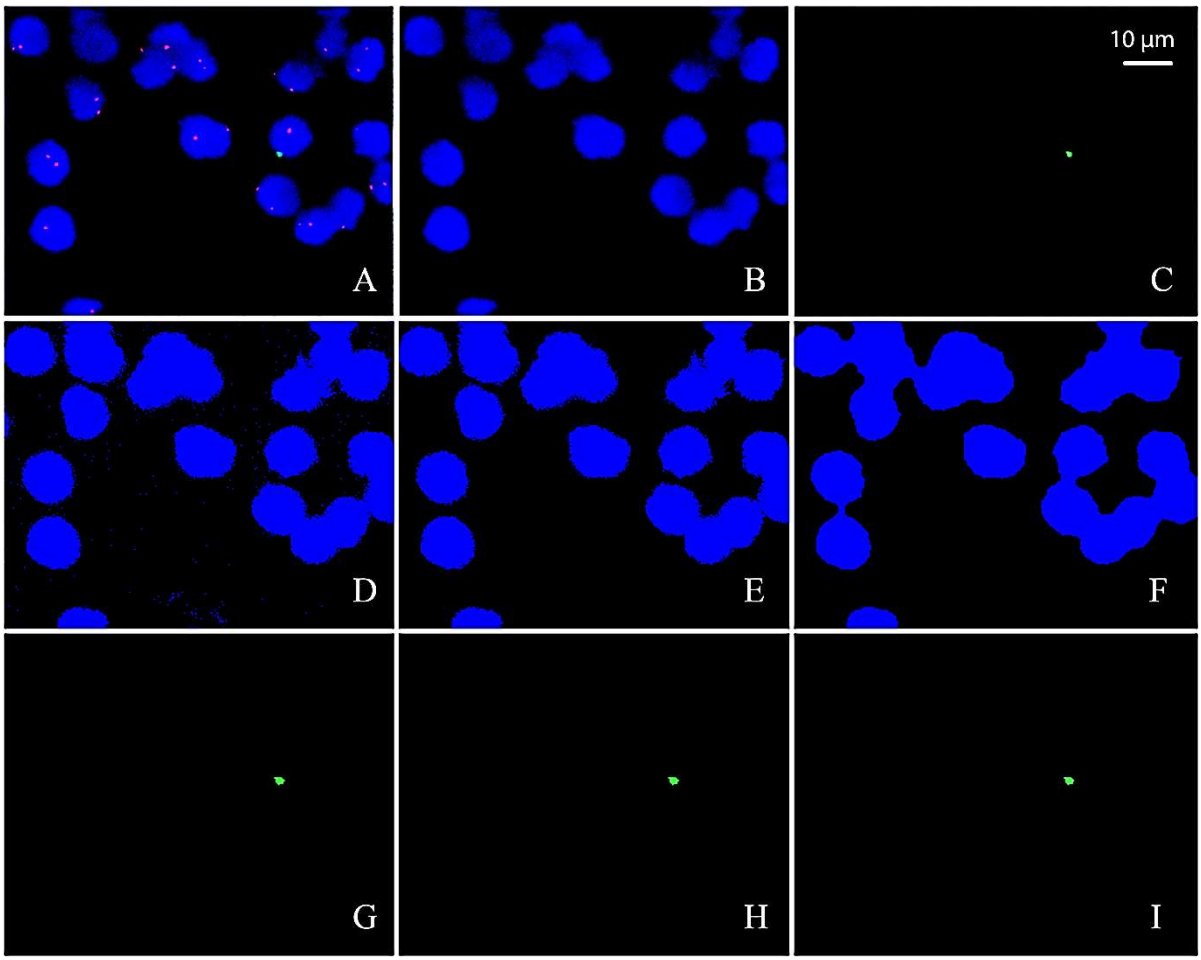

Figure 2. AxioVision Commander script for the automatic detection of male fetal cells: A. Original image, split up in B. DAPI image and C. SpectrumGreen image; D. Threshold interactive on the DAPI image: segmentation based on the definition of a brightness range. Pixels within the defined gray level range are set to the maximum gray value 1 (pseudocolor blue); whilst pixels outside it are set to the minimum gray value 0 (black), resulting in a binary image; E. Scrap of image D: removing all artefacts too small to be possibly originating from cell nuclei; F. Close: filling in gaps in the contours of the nuclei; G. Dynamic Threshold of the SpectrumGreen image, resulting in a binary image showing the Y chromosome FISH spots; H. Scrap of image G: all regions smaller than 10 pixels and larger than 65 pixels are removed; I. Masking of the binary images F and H: retaining only the SpectrumGreen FISH signals lying in a nucleus. In the last step, to be included as a true FISH signal, the detected regions had to fulfil four measurement parameter conditions with regard to area of the region, lowest and highest pixel density (fluorescence intensity/area²) and standard deviation of the pixel density (not shown).

Repeated FISH

Results of FISH were confirmed using another CEP Y FISH probe labelled with SpectrumAqua (Vysis). The repeated FISH protocol was performed as described by Liu *et al.* with a few minor modifications [23]. Cover slips applied after FISH were washed off in water. Slides were incubated for 10 min in respectively 60% formamide/2x SSC solution; 2x SSC and 4x SSC/0.1% NP-40 solution at 50°C. Slides were dehydrated for 1 min through an ethanol series (70%, 90% and 99%) at RT and air-dried.

Denaturation, hybridization and subsequent washing steps were performed as described above. Results of the repeated FISH were visualized by using the Zeiss filter set no. 47 (BP 436/20, FT 455, BP 480/40) (**Figure 1 B**).

Phenotyping of the fetal microchimeric cells

For 6 patients with an AITD, phenotyping of the male fetal microchimeric cells was performed. After isolation of the PBMCs, B, $CD4^+$ T and $CD8^+$ T cells were enriched using an EasySep positive selection strategy (Stemcell Technologies, Vancouver, Canada) according to manufacturer's instructions. These cell fractions were cytospun on poly-L-lysine slides and underwent FISH and repeated FISH as described above. Purity of the different isolated fractions was determined by flow cytometry. Cells were stained with monoclonal antibodies against CD3 (labelled with PE-Cy5; 1:20), CD4 (FITC; 1:20), CD8 (PE-Cy7; 1:20) and CD19 (PE; 1:20) (eBiosciences) for 30 min on ice, in the dark [24]. All analyses were performed on a Cytomics FC500 flow cytometer (Beckman Coulter, Miami, Florida, US) and data analysis was performed by CXP analysis software (Beckman Coulter).

Statistical analysis

Levels of significance were calculated by SPSS (IBM, New York, US) using Mann Withney (MW) test. $p < 0.05$ was regarded as significant.

Results

Study participants

A medical history concerning former pregnancies, transplantations and blood transfusions, which can influence the results of microchimerism, was available for patients (**Table 1**) and healthy volunteers (**Table 2**). Patients with an AITD and healthy volunteers, both with a son, were of similar age (mean 32.1 yr (range 25-37) for patients and mean 31.1 yr (range 26-39) for healthy volunteers; $p = 0.41$). Three healthy volunteers who never were pregnant and two healthy volunteers who were pregnant at the conduct of the study, were younger (mean 26.4 yr (range 25-27 yr); $p = 0.015$). Patients and healthy volunteers with son, had similar numbers of children (mean 1.4 (1-2) versus 1.5 (1-2); $p = 0.69$) and similar number of boys (mean 1 versus 1.2 (1-2)); which were of similar ages (mean 32.4 months (10-68 months) versus 36.3 months (3-91 months); $p = 0.85$). None of the patients and healthy volunteers had any other disease of the thyroid. There was no significant difference in the amount of isolated PBMCs/ml PB between patients and healthy volunteers (data not shown).

Table 1: Patients' information possibly relevant to fetal microchimerism (*within the conduct of the study)

Patient	AITD	Age patient (years)	Diagnosis since birth of youngest son (months)	Age (months)* and sexes of the children	Miscarriage	Transfusion	Additional information considering the thyroid	Male fetal cells/ 1.000.000 maternal cells
1	HT	32	10	10; male	-	-	-	**11**
3	HT	35	12	39; male, 16; female	3 x very early in pregnancy	-	Aunt with thyroid dysfunctions	**8**
5	HT	25	8	16; male	-	-	-	**8**
6	HT	35	3	51; male, 87; female	5 yrs ago	-	-	**7**
7	HT	36	4	26; male	-	-	Mother with hypothyroid	**7**
9	HT	29	unknown	15; male	-	-	-	**9**
11	HT	32	unknown	18; male	2 x, 1 and 3 years ago	-	Mother and grandmother with hypothyroid	**10**
2	GD	37	12	37; male	6,5 months old death born son 4 yrs ago	-	Aunt with hyperthyroid	**29**
4	GD	32	4	25; male	-	9 yrs ago	-	**15**
8	GD	32	unknown	68; male, 8; female	-	-	-	**14**
10	GD	28	5	37; male, 123; female	-	-	-	**21**

Table 2: Healthy volunteers' information possibly relevant to fetal microchimerism.

Healthy Volunteer	Age volunteer (years)	Age (months)* and sexes of the children	Miscarriage	Transfusion	Additional information considering the thyroid	Male fetal cells /1.000.000 maternal cells
1	31	43; male	1.5 yr ago at 7 weeks	-	-	**3**
2	39	91; male, 59; male	-	-	-	**3**
3	34	45; male, 20; male	-	-	Sister with possibly GD	**3**
4	26	6; male	-	-	-	**1**
5	29	54; male	-	-	-	**1**
6	29	33; male, 7; female	17m ago	-	-	**1**
7	32	32; male, 68; female	-	-	-	**5**
8	31	50; male, 89; female	-	-	-	**1**
9	33	3; male	2x; 2 and 1 yr ago	-	-	**0**
10	27	6; male	-	-	-	**1**
Pregnant 1, 1 week before delivery	27	-	-	-	-	**2**
Pregnant 1, 1 week postpartum	27	0; male	-	-	-	**2**
Pregnant 1, 6 months postpartum	28	6; male	-	-	-	**1**
Pregnant 2, 1 week before delivery	26	-	-	-	-	**1**
Pregnant 2, 1 week postpartum	26	0; male	-	-	-	**1**
Pregnant 2, 6 months postpartum	26	6; male	-	-	-	**1**
Negative control 1, never pregnant	26	-	-	-	-	**0**
Negative control 2, never pregnant	25	-	-	-	-	**0**
Negative control 3, never pregnant	27	-	-	-	-	**0**

*within the conduct of the study

Male fetal microchimerism in patients and healthy volunteers

The number of male fetal cells detected in patients with HT or GD and healthy volunteers is shown in **Table 1** and **2** respectively. All patients had detectable male fetal microchimerism in their PB, ranging from 14 to 29 male fetal cells per million maternal cells for patients with GD and from 7 to 11 male fetal cells per million maternal cells for patients with HT. In all healthy volunteers who gave birth to a son, except for one, male fetal microchimeric cells were found in PB and ranged from 1 to 5 male fetal cells per million maternal cells. There was a statistically significant difference between patients with GD and patients with HT compared to healthy controls (respectively p = 0.002 and p = 0.0007, MW) (**Figure 3**). Moreover, patients with GD had significant more male fetal cells in their blood compared to patients with HT (MW, p = 0.0061).

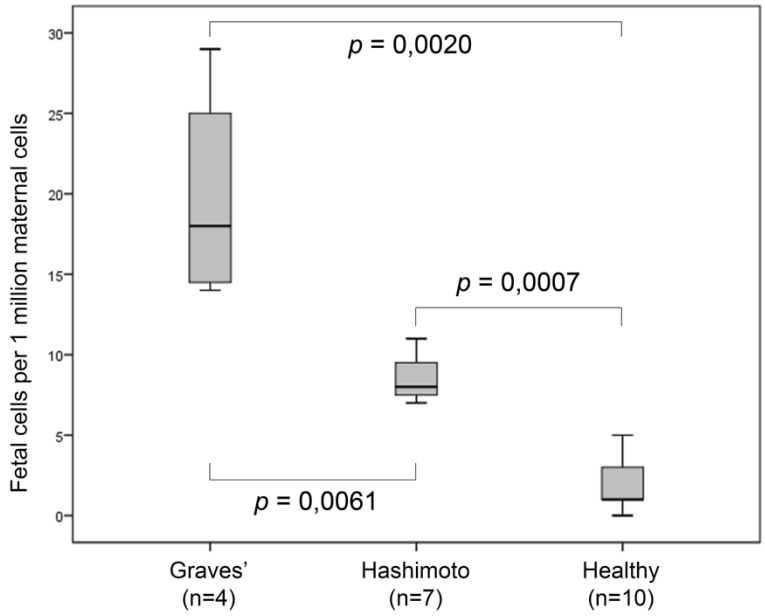

Figure 3. Boxplot: Male fetal cells in patients with GD or HT, and healthy volunteers with son: Minimum and maximum numbers of detected fetal cells are shown, as well as first quartile, median and third quartile. The number of fetal cells was significantly different between the three groups (p <0,05). Moreover, a significant difference between patients with GD and patients with HT was observed (MW, p = 0,0061).

Blood obtained from two women pregnant of a boy, contained respectively 2 and 1 male fetal cell(s) per million maternal cells. One week postpartum, these women had the same amount of male fetal cells in their blood as before delivery (2 respectively 1). Blood was also obtained 6 months postpartum and revealed in both women 1 male fetal cell per million maternal cells (**Table 2**).

As every woman had given birth to a son, the origin of the male cells is likely to be fetal. However, persistent microchimerism occurring after blood transfusion or transplantation has also been described [25], and this possibility cannot be ruled out for patient 4 (GD) who had a blood transfusion 9 years ago. However, as the results of this patient did not differ from the results of other

patients with Graves' disease, and hence inclusion or exclusion didn't influence the results, this patient was included in our study but results for this specific patient should be interpreted carefully.

The negative control group, consisting of three women who had never been pregnant nor had a transfusion or abortion, was consistently negative for male cells (**Table 2**).

Flow cytometry for determination of purity of EasySep isolated cells

Purity of the EasySep isolated cells was assessed by flow cytometry (Beckman Coulter Cytomic FC 500). For the isolation of B cells, $CD4^+$ T cells and $CD8^+$ T cells, a purity of respectively 97.6%, 97.4% and 94.1% was obtained (data not shown).

Fetal microchimeric cells in PBMC subsets

All patients selected for our analysis were positive for male cells in unsorted PBMCs. **Table 3** shows the distribution of the male fetal cells in the different cell subtypes ($CD4^+$ T, $CD8^+$ T, B cells and other cell types) for 6 patients with an AITD (patient 2, 7, 8, 9, 10 and 11). For patient 5, only male fetal T cells were detected (9.3 fetal cells per 1 million maternal T cells) and no subdivision into $CD4^+$ and $CD8^+$ T cells could be made, due to sample amount limitation. No male fetal B cells or other male fetal cell types were detected in this patient (data not shown in Table 3).

In patients with GD, the majority of the male fetal cells were found in the B cell fraction and $CD4^+$ T cell fraction while in patients with HT, male fetal cells were mainly $CD8^+$ cytotoxic T cells.

Table 3: Male fetal cells in the sorted cell fractions in patients with HT (patient 7, 9 and 11) and GD (patient 2, 8 and 10).

Cell type		Pat 7 (HT)	Pat 9 (HT)	Pat 11 (HT)	Pat 2 (GD)	Pat 8 (GD)	Pat 10 (GD)
B cell	**$CD19^+$**	0	3.4	1.2	4.0	4.0	9.0
T cell	**$CD4^+$**	0	1.5	1	1.0	2.5	9.7
	$CD8^+$	4.7	6.0	3.4	1.5	0.5	9.3
Other cell types		0	0	0	0	0	9.3

Data are represented as normalized counts of male fetal cells per 1 million maternal cells. T cells were split up immediately into CD4+ and CD8+ T cells. B and T cells were positively isolated with the EasySep® isolation kits. Cells not isolated with T or B cells, formed the cell population 'other cell types'. B cells were counted in a range from 1.000.000 to 2.000.000 cells, $CD4^+$ T cells ranged from 1.000.000 to 2.250.000 and $CD8^+$ T cells from 1.500.000 to 2.000.000.

Discussion

Hashimoto's thyroiditis and Graves' disease, two autoimmune thyroid diseases, occur more often in women than in men and show an increased incidence in the decades that follow parturition [10,17,19,20]. It has been hypothesized that fetal microchimeric cells play a role in the pathogenesis of these diseases [5,26,27]. Although male fetal microchimerism has already been shown in the thyroid glands in 50% of these patients, no studies to date have detected and characterized fetal cells in peripheral blood, although blood from these patients is easier to obtain in contrast to thyroid tissue [9,11,26,27]. Studies describing the presence of fetal cells in thyroid glands, often do not mention the reason of removal of the thyroid gland [9,27]. Srivatsa *et al.* analysed thyroid glands from patients with HT removed because of follicular neoplasm or papillary carcinoma [11]. The presence of tumor cells may confound the analysis and the results [9,27,28]. Therefore, only patients without any other disease of the thyroid were included in our study. Our study focused only on women who had given birth to a son since male fetal cells are easier to detect in contrast to female fetal cells.

To examine the presence of male fetal cells in autoimmune thyroid diseases, blood from female patients with GD or HT who had given birth to a son maximum five years before analysis, was assessed in this study. Using FISH and repeated FISH as an additional confirmation, the number of male fetal cells in patients was compared with that detected in healthy volunteers. All patients with an autoimmune thyroid disease included in our study had detectable male fetal microchimerism in their blood. This is contrary to results obtained in the thyroid glands where only 50% of the patients had male fetal microchimerism. The highest number of male fetal cells was observed in the unsorted PBMC fraction of patients with GD (14 to 29 male fetal cells per million maternal cells), followed by HT (7 to 11) compared to the low number of male fetal cells detected in healthy volunteers (0 to 5). This indicates a higher degree of male fetal microchimerism in AITD compared to healthy controls. Moreover, significant more male fetal cells were detected in patients with GD compared to patients with HT ($p = 0.0061$).

Two additional control groups were included in our study. Analysis of the blood of two pregnant women revealed respectively 1 and 2 male fetal cells per million maternal cells. This corresponds to the number of male fetal cells detected in our healthy group and the amount detected by Bianchi *et al.* [6]. As a negative control group for this study, blood from women who never were pregnant nor had a transfusion or transplantation, was obtained. No male cells were detected in their blood, which gives strong evidence of the reliability of the techniques used in our study.

As a significant difference in male fetal cells was found between patients with HT and GD, it can be presumed that fetal cells have a different role in the pathogenesis of both diseases. Our study focused on the presence of male fetal B and T cells because these subsets are more likely to initiate or be involved in immune response. In patients with HT, mainly male fetal $CD8^+$ cytotoxic T cells were found. In patient 5, only male fetal T cells were detected (9.3 male fetal cells per million maternal cells). In patient 7, male fetal cells were only detected in the $CD8^+$ T cell fraction (4.7 male fetal cells per million maternal cells). In patient 9 and 11, the majority of male fetal cells was composed of $CD8^+$ T cells (respectively 6 and 3,4 male fetal cells per million maternal cells). The remaining male fetal cells for these patients consisted of $CD4^+$ T cells (respectively 1.5 and 1 male fetal cell(s) per million maternal cells) and B cells (respectively 3.4 and 1.2 male fetal cells per million maternal cells). One might speculate that these cytotoxic T cells could cause cell death leading to hypothyroidism [29]. In GD however, the majority of male fetal cells was found in the B cell fraction (4 male fetal cells per million maternal cells for patient 2 and patient 8). These B cells could possibly be activated by fetal $CD4^+$ T cells (1 male fetal cell per million maternal cells and 2.5 male fetal cells per million maternal cells respectively for patient 2 and 8). In one patient with GD (patient 10), more male fetal T cells (9.7 male fetal $CD4^+$ T cells per million maternal cells and 9.3 male fetal $CD8^+$ T cells per million maternal cells) than male fetal B cells (9 male fetal cells per million maternal cells) were found, along with some other cell types (9.3 male fetal cells per million maternal cells). These other cell types were cells not isolated during selection of the T and B cells and are likely to be natural killer (NK) cells or hematopoietic progenitor cells capable of differentiating into immune competent cells [30]. One might speculate that thyroid-reactive T cells could cause activation of thyrotropin receptor (TSHR)-reactive B cells, secreting TSHR-stimulating antibodies causing hyperthyroidism [29]. These thyroid antibodies have already been described in blood [31].

Fetal microchimeric cells could play a role in the pathogenesis of AITD in two ways: direct or indirect. In a direct manner, fetal lymphoid cells migrating into the thyroid could initiate a graft-versus-host reaction against maternal thyroid antigens resulting in an autoimmune thyroid disease [5,13]. On the other hand, intrathyroidal fetal cells, not necessarily of the lymphoid lineage, could indirectly be involved in the pathogenesis of autoimmune thyroid disease by activating intrathyroidal maternal T cells against fetal antigens. Despite the fact that the male fetal cells were only characterized in a limited number of patient samples, an increase in male fetal lymphocytes was clearly shown, which provides support for the first hypothesis.

Conclusion

In conclusion, our findings indicate a significant difference in number of male fetal cells in the maternal circulation of patients with an AITD and healthy volunteers despite similar age, number and gender of their children. In addition, a significant difference was found between patients with GD and HT, where patients with GD had more male fetal cells in their blood circulation. Moreover, the male fetal cells were of a different cell type which might possibly correlate to the pathogenesis of both diseases. Our study shows a clear association between male fetal microchimeric cells and autoimmune thyroid diseases and indicates the value and need for further research in this field.

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CHAPTER 5

Fetal microchimerism in autoimmune thyroid diseases: harmful, beneficial or innocent for the thyroid gland?

Invited review

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Abstract

Autoimmune thyroid diseases (AITD) show a female predominance, with an increased incidence in the years following parturition. Fetal microchimerism has been suggested to play a role in the pathogenesis of AITD. However, only the presence of fetal microchimeric cells in blood and in the thyroid gland of these patients has been proven, but not an actual active role in AITD. Is fetal microchimerism harmful for the thyroid gland by initiating a graft-versus-host reaction (GvHR) or being the target of a host-versus-graft reaction (HvGR)? Is fetal microchimerism beneficial for the thyroid gland by being a part of tissue repair or are fetal cells just innocent bystanders in the process of autoimmunity? This review explores every hypothesis concerning the role of fetal microchimerism in AITD.

Autoimmune Thyroid Diseases

Autoimmune thyroid diseases (AITDs) are the most prevalent autoimmune disorders affecting up to 5% of the general population [1,2]. The two most common forms of AITD are Graves' disease (GD) and Hashimoto's thyroiditis (HT). Although HT and GD are multifactorial diseases where genes and environmental factors influence the onset and development of the disease [3], fetal microchimeric cells have been proposed to play a functional role in the pathogenesis of HT and GD [4-10].

Fetal microchimerism in AITD

Fetal cells have shown to be more common in thyroid glands [6-10] and in blood [4,9] of patients with AITD compared to healthy controls or patients with a benign adenoma or nodular goiter. Results of these studies are shown in **Table 1**.

Table 1: Studies describing fetal microchimerism in AITD

Author	(Autoimmune) Thyroid Disease or Healthy	Biological material	Technique	% of women positive for fetal cells (n/total)	Number of fetal cells/number of maternal cells
Lepez et al. [4]	HT	Blood	FISH and repeated FISH	100% (7/7)	7-11 /1.000.000
	GD			100% (4/4)	14-29 /1.000.000
	Healthy			90% (9/10)	0-5 /1.000.000
Renne et al. [7]	HT	Paraffin-embedded thyroid tissue	FISH	60% (15/25)	1-6/section, CD45+ (no thyrocytes)
	GD			40% (6/15)	ND
	Thyroid adenoma			22% (2/9)	ND
Klintschar et al. [6]	HT	Paraffin-embedded thyroid tissue	Qualitative PCR *SRY* and Amelogenin	47% (8/17)	ND
	Nodular goiter			4% (1/25)	ND
Klintschar et al. [10]	HT	Paraffin-embedded thyroid tissue	Qualitative PCR of *DYS14* of Y chromosome	38% (8/21)	15-4900/100.000
	Multinodular goiter			5% (1/18)	182/100.000
	Healthy			0% (0/17)	ND
Ando et al. [9]	GD	Paraffin-embedded thyroid tissue	PCR-ELISA *SRY*	20% (4/20)	ND
	Thyroid adenoma			0% (0/6)	ND
	GD	Fresh-frozen thyroid tissue		85% (6/7)	14-295/100.000
	Thyroid adenoma			25% (1/4)	17/100.000
	GD	Blood		47% (8/17)	1-10/100.000
	Healthy			28% (4/14)	1-87/100.000
	Polycystic ovary syndrome never pregnant			0% (0/16)	0/100.000
Srivatsa et al. [8]	Various thyroid disorders, without documented male children	Paraffin-embedded thyroid tissue	FISH	44% (4/9)	1-165/section, individual or in cluster
	Various thyroid disorders, with male children			63% (12/20)	
	Healthy controls			0% (0/8)	ND

ND: not determined

However, as shown by Ando et al. [9], data obtained on paraffin-embedded tissue are difficult to compare with data obtained on fresh-frozen material as paraffin-embedded tissue is subject to DNA-fragmentation. Moreover, techniques used to study fetal microchimerism, Fluorescence *in situ* Hybridisation (FISH), qualitative and quantitative PCR, have different sensitivities influencing the results of the studies [5,9].

In patients without history of male full-term pregnancy, male microchimerism has been found in blood and in the thyroid gland [9,11]. Abortion or undetected miscarriage can however also lead to microchimerism as transfer of microchimeric cells starts at the fourth week of pregnancy [12]. Studies investigating the occurrence of unrecognized miscarriages have reported that the rate of pregnancy loss prior to the first missed period is approximately 22-30% [13]. Moreover, other sources of microchimerism, natural and iatrogenic, have been described. Not only do fetal cells cross the placenta and enter into the maternal circulation during pregnancy, but due to a bidirectional transfer between the mother and the fetus, maternal cells can also enter the fetal circulation. The latter has been described in tissues of patients with different diseases such as type 1 diabetes [14-16]. Other naturally acquired sources of microchimerism include feto-fetal transfer from an undetected vanishing twin [17], or possibly from an older sibling. Iatrogenic sources of microchimerism include blood transfusion and organ transplantation [18]. Results of studies describing fetal microchimerism in AITD must therefore be interpreted carefully.

Fetal microchimeric cells in autoimmune thyroid diseases: harmful, beneficial or innocent for the thyroid gland?

The consequences of long-term persistence of fetal microchimerism are difficult to assess to date. As fetal microchimerism in peripheral blood is an almost universal finding during normal pregnancy [19] and during the postpartum period [4,20], the presence of fetal cells in the circulation does not indicate an aberrant immune response by the mother. An actual active role of fetal cells in autoimmune diseases has not yet been proven, only the presence of these cells in tissues affected by the disease. The mechanism that attracts fetal cells to migrate into the maternal thyroid gland has not yet been studied. Cytokines, chemokines, and adherent factors may be involved [21].

There are a number of potential mechanisms by which fetal immune and non-immune cells may influence the autoimmune status of the mother. Harmful, beneficial as well as innocent effects have been assigned to the long-term persistence of fetal microchimeric cells [22]. In a harmful way, fetal cells can cause autoimmune thyroid disease by initiating a graft-versus-host reaction (GvHR), or the maternal host can initiate a host-versus-graft reaction (HvGR) against intrathyroidal fetal cells. In a

beneficial way, fetal cells can offer help in tissue repair. Another hypothesis suggests that fetal cells are innocent bystanders in the thyroid gland and do not participate in the autoimmune reaction.

Harmful

It is possible that fetal cells themselves play an active role in determining the maternal immune repertoire. Postpartum, fetal immune cells can interact with maternal cells and may initiate the onset of postpartum AITD [23,24]. Presence of fetal cells in the thyroid gland [6-10], where the autoimmune reaction is taking place, supports this hypothesis.

Fetal cells could act as effector cells initiating a GvHR (hypothesis 1) or could be target of an HvGR (hypothesis 2). After delivery, when placental immune suppression is lost, fetal immune cells may become activated and initiate an autoimmune reaction based on HLA dissimilarities. The activation of fetal immature T cells, monocytes, macrophages and NK cells and the production of inflammatory cytokines and chemokines are believed to initiate the autoimmune disease (hypothesis 1) [25,26]. Alternatively, fetal cells could be recognized as partially allo-immune and give rise to an autoimmune reaction by a direct response of the maternal cells to the fetal cells or by molecular mimicry between fetal antigens and intrathyroidal maternal antigens (hypothesis 2) [27-29] .

Hypothesis 1: Microchimerism induces a graft-versus-host reaction (GvHR)

During pregnancy, circulating fetal cells do not initiate disease, which indicates the success of placental immune suppression. After pregnancy, once maternal tolerance against fetal cells is lost [23], fetal cells could become activated due to an unknown factor. Triggers can be viral or bacterial agents, drugs or abnormal tissue proteins [30]. Accumulating evidence suggests that these activated fetal microchimeric cells initiate a local immune GvHR. Similarities between autoimmune diseases and graft-versus-host disease (GvHD) suggest a functional role for fetal microchimerism to initiate a GvHR.

To be able to initiate a GvHR, three conditions must be fulfilled [31]. First of all, fetal cells have to be present at the site of the immune reaction. As fetal cells have already been detected in blood [4,9] and thyroid glands of patients with AITD [6-10], this condition is fulfilled. Secondly, the microchimeric cells must be immunologically competent T or B cells. In blood of patients with HT, mainly fetal $CD8^+$ cytotoxic T cells were detected [4]. In our opinion, these fetal cytotoxic T cells could cause cell death leading to hypothyroidism [32]. In patients with GD, the majority of fetal cells was found in the B cell fraction [4]. These B cells could possibly be activated by fetal $CD4^+$ T cells, also detected in the blood of these patients. From our point of view, it can be presumed that fetal thyroid-reactive T cells could

cause activation of B cells, secreting TSHR-stimulating antibodies causing hyperthyroidism [32]. Thyroid auto-antibodies have already been described in blood [33]. These mechanisms are illustrated in **Figure 1, upper left**.

Even fetal progenitor cells have been shown in maternal blood and tissues subject to autoimmunity [34-36]. It is possible that these progenitor cells develop in the maternal thymus and bone marrow into respectively functional T and B cells, as shown in mice [37], and migrate into the maternal thyroid gland where they start an immune reaction. However, some level of HLA compatibility is necessary to survive positive and negative selection in the thymus and periphery, which has been shown in systemic sclerosis (SSc) [20]. Moreover, the presence of fetal $CD3^+$ T cells [26] and $CD4^+$ T cells [38] has been proven, which leads to the hypothesis that fetal cells could start an immune reaction against maternal cells.

Thirdly, microchimeric cells must recognize the cells of the host as foreign. In patients with SSc, in contrast to controls, CD28 stimulation of PBMCs caused an increase in the amount of fetal cells [39]. T cell clones have been isolated from female patients with SSc which turned out to be self-reactive and appeared to be male in origin. These clones produced higher concentrations of IL-4 than control clones did [40]. These results suggest that the fetal cells were immunologically active and able to proliferate, and that one of the immune targets of these fetal immune cells were maternal antigens. This has to be confirmed for AITD.

An argument against this hypothesis is the low concentration of the fetal cells in the maternal circulation [4,6], and that only a part of all patients with AITD show microchimerism in their thyroid gland [6-10]. In patients who appear to be negative however, it is possible that fetal microchimeric cells are not detectable by the methods used, that microchimeric cells originate from another (natural or iatrogenic) source or that the clinical context of the studied samples is not properly described. It is also possible that only female fetal cells, provoking the immune reaction, are present [6]. Our study of microchimerism in blood of patients with AITD however, showed male fetal cells in all patients with an AITD. Taken together, these data suggest a potential role of fetal cells in the pathogenesis of AITD [4,6].

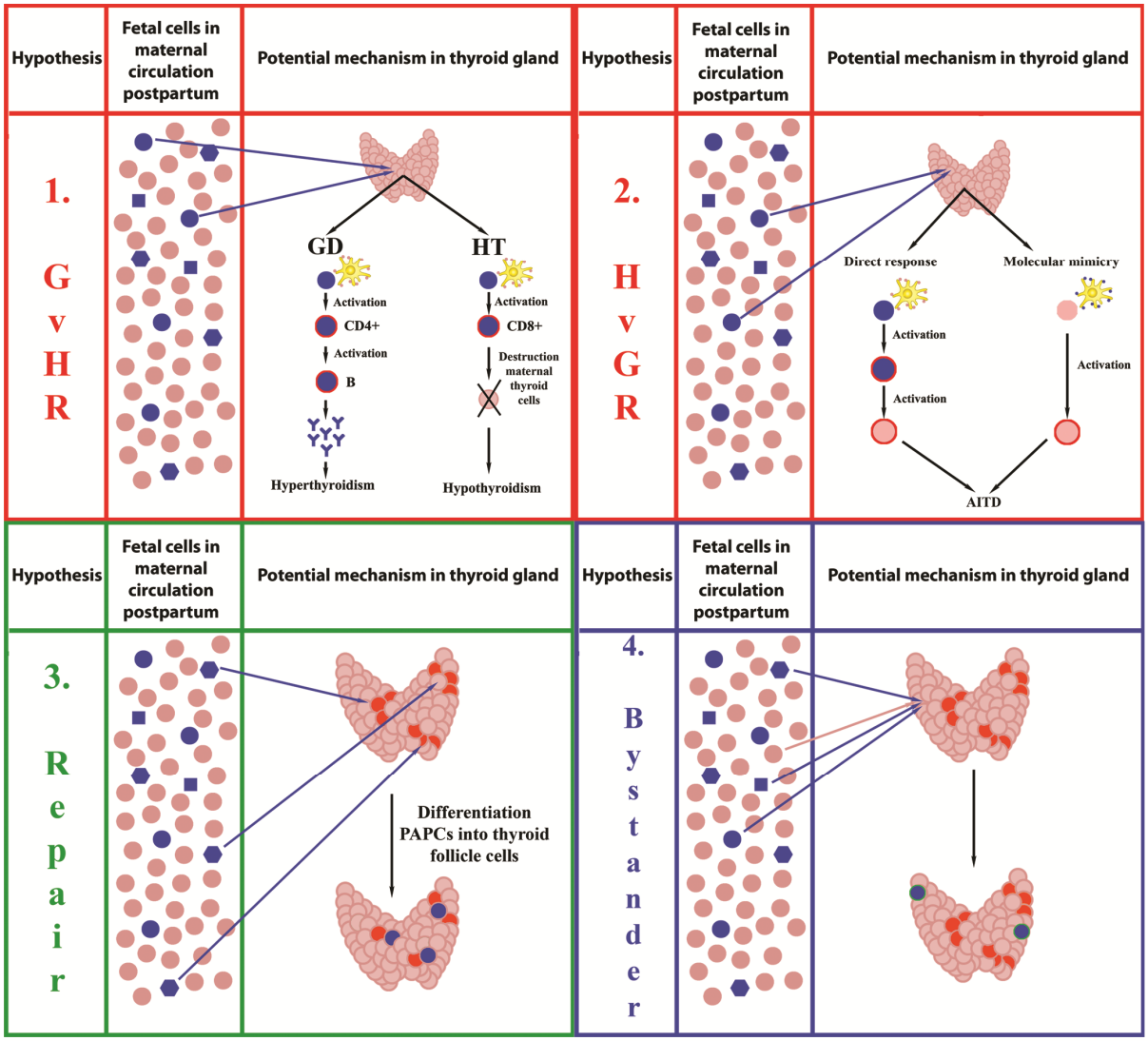

Figure 1: Potential mechanisms of harmful (red), beneficial (green) and innocent (blue) microchimerism in the thyroid gland.

Hypothesis 2: Microchimerism induces a host- versus-graft reaction (HvGR)

Alternatively, fetal cells could initiate AITD by being the target of a HvGR [27]. Fetal microchimeric cells have to be recognized by the mother as foreign. Because fetal cells contain paternal genes, the cells are semi-foreign to the mother. After delivery, once the immune tolerance mechanisms of the mother are no longer present, maternal cells may react against the paternal antigens of the intrathyroidal microchimeric cells [6,8,10]. Maternal cells could induce an immune reaction by a direct response to microchimeric cells or by cross-reactivity due to molecular mimicry [41], both illustrated in **Figure 1, upper right**.

In case of a direct response to microchimeric cells, fetal cells can initiate a GvHR against maternal antigens upon which intrathyroidal maternal autoreactive T cells become activated which eventually leads to the maternal cells causing damage to the tissue. Another possibility is that fetal antigen presenting cells present maternal antigens to maternal immune cells resulting in an immune reaction from the mother against her own cells [42]. In molecular mimicry, maternal cells start an immune reaction against fetal antigens, but due to similarities between fetal antigens and maternal thyroidal self-antigens, autoimmunity to the thyroid occurs [41].

Evidence for this HvGR hypothesis is shown in morphea or localized scleroderma, where fetal microchimeric cells were mainly displaying an antigen-presenting role as B cells and dendritic cells [43]. In addition to this study, the maternal immune system is directly responsive to fetal cells since the level of microchimeric cells seems dependent on the level of fetal-maternal compatibility, at least in animal models [44].

Beneficial

An argument against a harmful role of microchimeric cells is the fact that fetal cells have also been detected in healthy women without signs of autoimmunity [7,8,45]. Male cells have been detected in thyroid, lung, lymph node and skin in women with sons and in kidney, liver and heart in women with and without sons [46]. In the latter group of women, an unrecognized miscarriage of a male fetus could have occurred. Presence of fetal microchimeric cells in affected and healthy women suggests that these cells do not play a direct role in triggering maternal autoimmune disorders. Instead of causing an autoimmune reaction, they could be a part of tissue repair.

Hypothesis 3: Microchimeric cells repair injured tissue

Beneficial microchimerism has mainly been described in cancer [47-49]. The first study on fetal microchimerism in cancer was performed by Cha et al. [47] In this study, male cells were detected in cervical tissue of patients with cervical cancer. However, Gadi et al. [50] were the first to make an

association between the presence of fetal microchimerism and the influence of pregnancy in breast cancer. As breast cancer was less prevalent in parous women compared to nulliparous women, fetal microchimeric cells may reduce the risk of developing breast cancer. Also, fetal microchimerism was less present in peripheral blood of women with breast cancer than in healthy women [50]. According to Dubernard et al. [51], fetal cells are recruited from the peripheral blood into the damaged tissue to repair it if malignancies are developed during pregnancy. Fetal microchimerism has also been investigated in thyroid cancer, cervical cancer, lung cancer and melanoma [8,47-49,52,53]. The proposed role of fetal microchimerism in cancer has been a beneficial one, although a role in disease progression has also been considered as fetal cells can contribute to lymphangiogenesis or tumor growth [53]. In mice, fetal progenitor cells participate in inflammation and angiogenesis during wound healing [54].

If fetal cells do have a function in tissue regeneration, two conditions must be fulfilled: they have to migrate to the damaged area and they have to show plasticity. During pregnancy, fetal progenitor cells have been detected in maternal blood and tissues [36,55]. They are capable of engrafting into maternal bone marrow [56]. After pregnancy, fetal microchimeric cells expressing tissue specific markers have been found in maternal tissues, both healthy and affected [46]. In humans, fetal hepatocytes [34,57,58], cardiomyocytes [59], endothelial cells, bone and cartilage [60] and intestinal epithelium [34] have been detected. In animal models, fetal hepatocytes [61], kidney tubular epithelium [61], neurons [62] and glia [63], and cardiomyocytes [64] have been detected. These data suggest that fetal progenitor cells are capable of differentiating into tissue specific mature cells within injured maternal organs [34,65]. The diversity of cell types into which microchimeric cells can apparently differentiate, suggests that a very early stem cell type is involved, the pregnancy-associated progenitor cells (PAPCs) [34,66].

In patients with multinodular goiter, fetal epithelial cells were detected. 14% to 60% of these cells stained positively with cytokeratin, a marker of epithelial differentiation [34]. Fetal microchimeric cells were also observed in thyroid glands of patients with various thyroid disorders, such as adenoma and thyroid carcinoma, but were absent in necropsy specimens from normal thyroid glands. Fetal cells were detected both individually and in clusters. In one patient with a progressively enlarging goiter, fully differentiated male thyroid follicles closely attached to and indistinguishable from the rest of the thyroid were observed [8]. In some patients with papillary thyroid cancer, fetal microchimeric cells were detected in tumor tissue as single cells or in clusters [48]. Presence of microchimeric progenitor cells in the adult thyroid gland could be a potential source for tissue regeneration [28,67], as shown in **Figure 1, lower left**. In women with papillary thyroid cancer, the

prevalence of male DNA was reduced in peripheral blood compared to healthy women [67]. As in other cancers, the specific homing to the injured tissue may explain their reduced number in maternal peripheral blood.

Adapting the phenotype of the cells in the maternal tissue is however not sufficient to dedicate a role in tissue repair as its function has not yet been proven. Moreover, it can be hypothesized that after successful repair by chimeric cells, a HvGR to these microchimeric cells could still be induced at a later time, if an altered immunological response occurs [41].

Innocent

Hypothesis 4: Microchimeric cells as 'innocent bystanders'

A fourth hypothesis suggests that fetal microchimeric cells are innocent bystanders and do not participate in triggering or exacerbating AITD [3].

Reports mentioned that a certain disease activity threshold is necessary for the significant detection of fetal microchimeric cells, suggesting that their presence is a consequence and not a cause of the disease [66,68]. It is possible that the microchimeric cells are equally distributed throughout the body. If tissue damage occurs, fetal cells will be attracted due to inflammatory infiltrates and the level of microchimerism in the diseased tissue will be higher compared to that of the healthy tissue, which would imply that there is no relation to the pathogenesis of the disease itself [41]. The relationship between inflammation and the presence of microchimerism could be indicative of this theory [69]. Fetal intrathyroidal cells, even immune cells, could therefore be a reflection of an ongoing local immune reaction without active participation [70]. Due to damage to the blood vessels, fetal cells could leak out into the damaged tissue without having an active role in tissue damage or repair [12]. This has been shown in **Figure 1, lower right**. Because pregnancy is very common and autoimmune diseases are quite rare, it is likely that only certain subsets of microchimeric cells have pathogenic potential, while most of them are only innocent bystanders.

An argument in favor of this hypothesis is the fact that three large epidemiological community-based studies failed to demonstrate an association between pregnancy, parity, abortion, and the presence of thyroid autoantibodies or thyroid dysfunction [71-73]. Fetal cells would only be a remnant of pregnancy. In contrast, a case-control study indicated parity as a potential risk for AITD by showing higher thyroid autoantibody levels in women with previous pregnancies compared to non-parous women [74]. However, HLA compatibility between fetal and maternal cells might be a more crucial risk factor than the number of pregnancies in the initiation of the autoimmune reaction by fetal microchimeric cells [7].

Conclusion

Microchimerism might have harmful, beneficial or innocent effects for the mother depending on a number of factors including the origin of the microchimeric cells, type of cells acquired, tissue environment, type of malignancy, time elapsed since microchimerism acquisition and age of the recipient. HLA haplotype and degree of differences between mother and child have the potential to affect the balance of beneficial versus harmful consequences of microchimerism for the recipient [75]. In our opinion, the more fetal cells show similarities with the maternal cells, the more likely they have the potential to start a GvHR once they have been activated by yet undetermined mechanisms.

Whether the higher prevalence of microchimerism in thyroid autoimmunity is mere coincidence or is a marker for immune-mediated disease requires further investigation. Further research to characterize the fetal cells detected in blood and tissues is mandatory.

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PART III

Forensic applications

CHAPTER 1

Aim and outline

The Laboratory of Pharmaceutical Biotechnology, which is part of the Forensic Institute of Ghent University, performs forensic DNA analyses, mainly for the Belgian magistracy. Besides forensic DNA analyses, procedures to analyze forensic samples are continuously optimized and improved.

One of the first steps in the analysis of forensic evidence is the search for biological material, such as blood, sperm, saliva and hairs, that can subsequently be used for DNA profiling. In this part of the thesis, the aim was to optimize and validate a fast screening method to select hairs useful for DNA analysis and to optimize a method to visualize bloodstains on dark fabrics.

Human hairs are frequently recovered as forensic evidence as humans shed about 150 hairs daily [1,2]. The identification and comparison of human and animal hairs can be helpful in demonstrating physical contact with a suspect, victim, and crime scene. Formerly, the use of a microscope was considered to be the only reliable tool for the identification and microscopic comparison of the hair recovered from the crime scene. Today, nuclear and mitochondrial DNA analysis can provide additional information. The success rate of nuclear DNA analysis of hair roots found at a crime scene is however quite low and negative results of hair analysis are frequently reported [3,4]. The aim of this study was to develop a fast nuclear staining method to predict the success rate of DNA analysis of hairs in forensics (**Chapter 2**).

Blood is a common body fluid often detected on pieces of evidence found at crime scenes, especially at scenes of violent crimes. Before DNA profiling can be performed, the presence of a bloodstain needs to be made visible. Especially when blood is present on a dark background, e.g. a dark fabric, the stain might be invisible for the naked eye. Therefore, the use of a visualization assay was optimized and validated to be able to detect bloodstains on different fabrics. Moreover, the combination of this assay with several presumptive blood tests was evaluated (**Chapter 3**).

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CHAPTER 2

Fast nuclear staining of hair roots as a screening method for successful STR analysis in forensics

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Abstract

Human hairs are frequently recovered as forensic evidence as humans shed about 150 hairs daily. The success rate of STR profiling of these hair roots is however quite low and negative results of hair analysis are frequently reported. To increase the success rate of DNA analysis of hairs in forensics, nuclei in hair roots can be counted after staining the hair root with DAPI, a fluorescent and non-destructive nuclear stain. Two staining methods were tested in order to reduce the incubation time with DAPI: a longer method with two 1 hour incubations in respectively a DAPI- and a wash-solution, and a fast, direct staining of the hair root on microscope slides.

The two staining methods were not significantly different. The results of the STR analysis for both procedures showed that 20 nuclei are necessary to obtain at least partial STR profiles. When more than 50 nuclei were counted, full STR profiles were always obtained. In 96% of the cases where no nuclei were detected, no STR profile could be obtained as expected. However, 4% of the DAPI-negative hair roots resulted in at least partial STR profiles. Therefore, each forensic case has to be evaluated separately in function of the importance of the evidential value of the found hair. The fast staining method was applied in 36 forensic cases on 279 hairs in total.

To our opinion, this fast staining method of hair roots with DAPI can be used to increase the success rate of hair analysis in forensics. Hair roots containing any visible nuclei will be selected for STR analysis.

Introduction

The identification of biological samples collected at crime scenes is established by DNA typing of different Short Tandem Repeat (STR) loci. As humans shed about 150 hairs daily, hairs from the victim or from the putative offender are frequently found at crime scenes [1,2]. Microscopic analysis of the hair can identify the origin of the hair (human or animal), establish the nature or shape of the hair tip, pigment pattern and color, and determine whether the hair has a root or not [3]. However, microscopic comparison between hairs collected at a crime scene and reference hair is very labor intensive and in most cases, no reference hair is available for comparison. Moreover, collection of hairs from a suspect might take place many months, possibly years, after the crime. Hence, the characteristics of the reference hair sample may look quite different from the hairs shed at time of the crime. The discriminative power of microscopic analysis is thus relatively small.

In contrast, STR analysis of the hair root can identify the donor of the hair. In many forensic cases however, no reportable STR profiles are obtained from hairs collected at crime scenes [4,5]. This can be explained by the growth phase of the hair. Hairs with intact root in the mitotically active anagen growing phase consistently yield reportable STR profiles while the inactive, naturally shed hairs in the telogen phase rarely yield informative STR profiles [5-9]. Unfortunately, 95% of the hairs found at a crime scene are telogen hairs [8,9].

The aim of this study was to optimize and validate a fast, non-destructive, easy to perform and inexpensive screening method to select those hair roots useful for STR analysis. Nuclear DNA can be stained with haematoxylin [3], which is known to reduce DNA yield [10,11], or can be labeled *in situ* [12] which is very time-consuming. Nuclei in hair roots can also be stained overnight with 4',6-diamidino-2-phenylindole or DAPI, a fluorescent dye that binds strongly to A-T rich regions in DNA [8,13]. The aim of this study was to validate a shorter staining protocol with DAPI, based on the protocol described by Bourguignon et al. [8], and to evaluate the impact of the staining on subsequent STR profiling. Furthermore, the influence of forensic adhesive films, used to collect hairs at a crime scene, was investigated.

Materials and methods

Staining of hair roots with 1 hour incubation in DAPI (part I)

58 hairs (plucked or spontaneously shed hairs) were collected from 9 volunteers. Hair roots were isolated by cutting the hairs approximately 1 cm above the hair root and were individually put into sterile 1.5 ml microcentrifuge eppendorfs. 10 µl of a DAPI/DABCO-solution (1.6 mg DAPI (Sigma); 2.24 g DABCO (1,4-diazabicyclo (2,2,2) octane) (Sigma), 10 ml Tris-HCl 0.2 M; pH 7.4) and 90 µl glycerol (Sigma) was added to the hair root. After 1 hour incubation at room temperature in the dark, the hair root was removed from this solution and transferred to another microcentrifuge eppendorf. 10 µl of a wash-solution (2.24 g DABCO; 10 ml Tris-HCl 0.2 M pH 7.4) and 90 µl glycerol was subsequently added to the hair root. After 1 hour incubation, hair roots were removed from this wash-solution and put on UV-sterilized microscope slides cleaned with bleach and 70% ethanol. 10 µl of the wash-solution was added to the hair root and a coverslip glass was applied.

DAPI-staining of hair roots directly on microscope slides (part II)

As a preliminary test to evaluate whether the staining procedure could be shortened even further by performing the DAPI-staining directly on hair roots on microscope slides, 23 hairs (plucked or spontaneously shed hairs) were collected from 7 volunteers. Hair roots were isolated as described above and were put on microscope slides, upon which 20 µl DAPI/DABCO-solution was added to the hair root. A coverslip glass was applied and hair roots were immediately visualized under the fluorescence microscope.

To compare both staining methods, hair roots of 54 naturally shed hairs from 5 donors were stained directly on microscope slides (part II) upon which images were acquired. In a next step, hair roots were removed from the microscope slide and were stained again using the method described in part I. Images were again acquired. Both images of the same hair root were compared to each other.

Hairs on adhesive films

To investigate the influence of possible loss of nuclei due to the adhesive tape, 10 hairs plucked from 1 donor were collected using adhesive films from the tape lifting kit (distributed by National Institution for Criminalistics and Criminology, Belgium). These hairs were removed from the adhesive film and were stained directly with DAPI on microscope slides as described in part II of the staining.

Forensic cases

The screening method described in part II was applied on 279 hairs, collected in 36 forensic cases. Hairs were mainly collected from clothes and some from tape lifting kits applied on car seats.

Microscopic evaluation of the staining

Image acquisition was carried out with an AxioVert 200M inverted fluorescence microscope (Carl Zeiss), equipped with the AxioVision multichannel fluorescence module and an AxioCam MRm camera (Carl Zeiss). Cell nuclei were visualized using Zeiss filter set no. 49 (G 365 nm, FT 395, BP 445/50). Slides were screened at 10x or 20x magnification using a Carl Zeiss short distance Plan-Apochromat® objective [14]. Hair roots were examined across several focal planes in order to confirm whether nuclei were present. DAPI fluorescent blue spots showing the shape and size of the human follicular cells (~3 – 6 µm) were counted according to Bourguignon et al. [8].

DNA extraction

After microscopic evaluation, hair roots were removed from the microscope slide and transferred in a 1.5 ml microcentrifuge tube. 200 µl 5% Chelex®100 (Bio-Rad) was added to the hair root [15]. After vortexing for 10 s, samples were incubated overnight at 56°C in a Thermomixer (Eppendorf). The following day, samples were incubated at 100°C for 8 minutes. Finally, samples were centrifuged for 3 min at 14000 x g [16].

DNA amplification and detection

All samples were amplified using an in house developed multiplex of 14 short tandem repeat (STR) loci (D3S1358, TH01, D21S11, D18S51, vWA, D8S1179, TPOX, FGA, D5S818, D13S17, SE33, CD-4, D7S820 and D16S539) and amelogenin [16,17]. Amplified fragments were separated and analyzed by capillary electrophoresis using an ABI PRISM 3100 or 3500xL Genetic Analyzer equipped with Genemapper ID-X 1.2 software (Life Technologies). Peak height minimum thresholds were set at 100 relative fluorescence units (RFU). Each STR profile of an analyzed hair root was compared to the STR profile of the donor of the hair. Profiles were subdivided into full (all loci gave interpretable results), partial (result for one or more loci did not meet the minimum thresholds) or no profile.

Statistical analysis

Level of significance was calculated by SPSS (IBM, New York, US) using the McNemar test. A *p*-value <0.05 was regarded as significant.

Results and discussion

1 hour incubation in DAPI (part I)

58 hair roots stained with DAPI for 1 hour, were subdivided into 4 groups depending on the number of visible nuclei as shown in **Table 1**. An example of a hair root without visible nuclei is shown in Figure 1A while an example of a hair root with more than 50 nuclei is shown in Figure 1B.

Results of STR profiling of these 58 hair roots are shown in **Table 1**. If 20 or more nuclei were observed, at least partial profiles could be obtained. STR profiling of hair roots containing more than 50 nuclei resulted in full STR profiles. All 38 hair roots without any visible nuclei resulted in no STR profile.

Table 1: STR profiling of 58 hair roots stained with DAPI for 1 hour

Number of visible nuclei	Total root N	N roots with STR profile		
		Full profile	Partial profile	No
0	38	0	0	38
<20	4	2	0	2
20<n<50	4	3	1	0
>50	12	12	0	0

Direct staining of hair roots with DAPI on microscope slides (part II)

To investigate whether direct staining with DAPI could also be used to screen hair roots suitable for STR analysis, 23 hair roots were stained directly on microscope slides and images were acquired immediately afterwards. An example of a hair root without visible nuclei after direct DAPI-staining on microscope slides is shown in Figure 1C; Figure 1D shows a hair root with more than 50 nuclei.

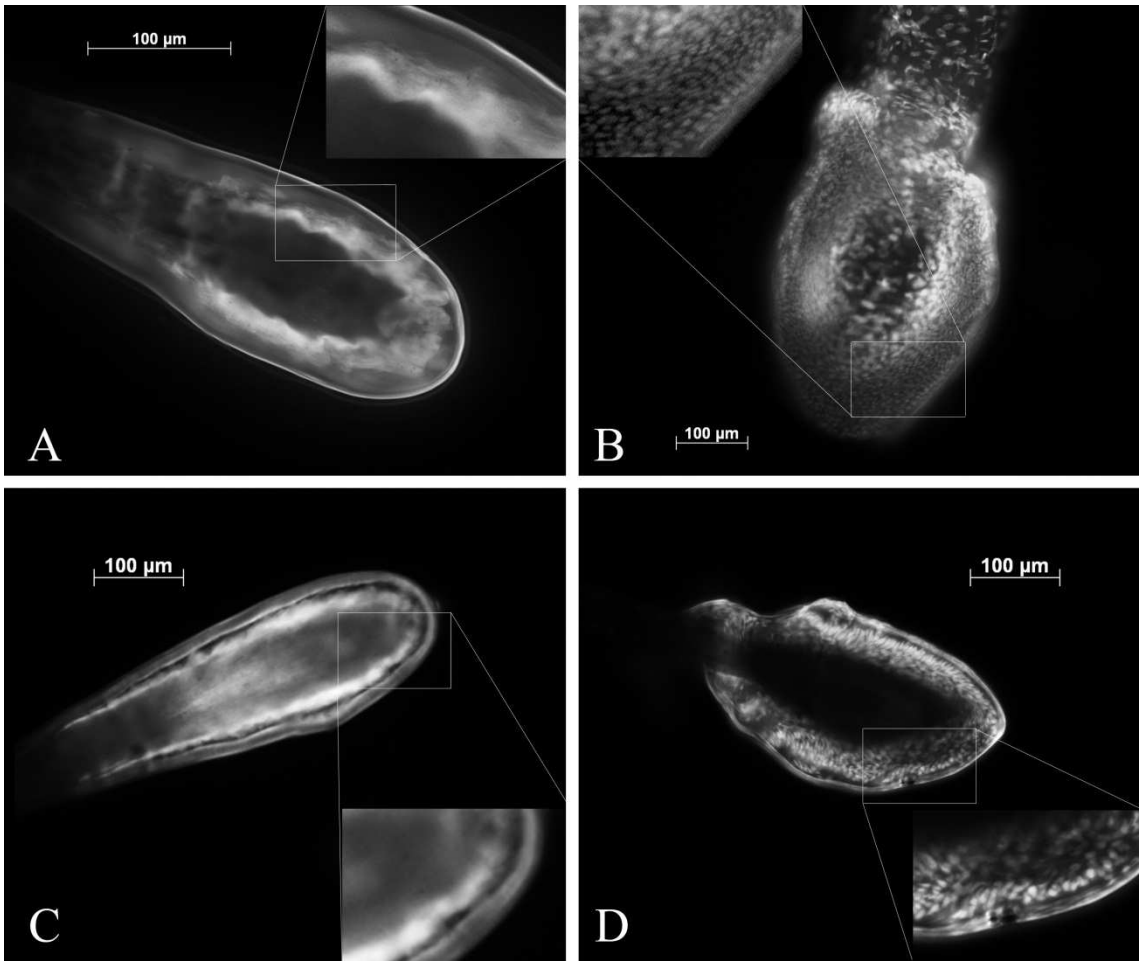

Figure 1: Hair root without visible nuclei (A) and with more than 50 nuclei (B) stained with DAPI for 1 hour (part I). Hair root without visible nuclei (C) and with more than 50 nuclei (D) stained directly on microscope slides (part II).

Results of the STR analysis of these 23 hair roots are shown in **Table 2**. The results of this preliminary test were comparable with those obtained after staining with one hour incubation. Even more, in all cases where nuclei were observed, full STR profiles could be obtained. All hair roots without visible nuclei resulted in no STR profile.

Table 2: STR profiling of 23 hair roots stained directly on microscope slides

Number of visible nuclei	Total root N	N roots with STR profile		
		Full profile	Partial profile	No
0	11	0	0	11
<20	1	1	0	0
20<n<50	5	5	0	0
>50	6	6	0	0

To be sure whether the immediate staining on the microscope slides would lead to the detection of the same number of nuclei compared to the staining with one hour incubation, the two staining

methods were performed on the same hair roots and compared. As nuclei of plucked hairs are immediately visible after direct staining of the hair root on microscope slides, focus has been put on naturally shed hairs, mimicking forensic situations. 54 hair roots of naturally shed hairs were first directly stained on microscope slides according to the protocol described in part II. After image acquisition, the same hair roots were stained again according to the protocol described in part I in which hair roots were incubated with DAPI for 1 hour and wash-solution for 1 hour. There were no significant differences between the two staining methods (McNemar test, p = 1.00), except for one hair. In this exception, direct staining of the hair root on a microscope slide resulted in detection of less than 20 nuclei, while staining with one hour incubation in DAPI resulted in detection of more than 50 nuclei. Evaluation of the staining and results of the STR analysis of these 54 hairs are shown in **Table 3**.

Table 3: Evaluation of the staining and STR analysis of 54 hair roots

Number of visible nuclei	Total root N after direct staining	Total root N after staining with 1h incubation	N roots with STR profile		
			Full profile	Partial profile	No
0	49	49	1	3	45
<20	5	4	4 (3[a])	0	1
>50	0	1	0 (1[a])	0	0

[a] number of roots with STR profile after staining with 1h incubation

Counting less than 20 nuclei, all hair roots but one resulted in full STR profiles. From the 49 hair roots without any visible nuclei, 3 resulted in a partial STR profile and 1 even in a full STR profile. 1 of the hair roots which resulted in a partial profile, showed presence of adhering material, presumably dandruff. Adhering material however, can contain DNA and could therefore result in a STR profile.

In an optimal situation, hair roots without visible nuclei could be discarded. In 96% (94/98) of all cases where no nuclei were observed, no STR profile was obtained. However, in 4% of these cases, a full or partial STR profile could be obtained. Therefore, results of DAPI-staining should always be considered in function of the importance of the evidential value of the found hair. If the hair is the only biological evidence in the forensic case, one might consider to submit the hair to STR analysis anyway, even if the staining is considered to be negative. If necessary, multiple hair roots can be pooled for STR analysis. In case the hair root did not yield a STR profile, the remainder of the hair can still be submitted to mitochondrial DNA analysis [18,19]. However, as STR analysis has a higher discriminative power compared to mitochondrial DNA analysis, the former is preferred.

Hairs on adhesive films

Ten hairs plucked from 1 donor were collected using the tape lifting kit, subsequently removed from the adhesive film and directly stained on microscope slides. In 8 of 10 cases, 21 to 50 nuclei were counted while in the remaining 2 cases, more than 50 nuclei were observed. In all cases, full STR profiles were obtained (data not shown). However, loss of nuclei after removing the hair root from the adhesive film could be observed as the adhesive film was re-examined under the fluorescence microscope and nuclei were found on the tape.

Therefore, if adhesive films are used for collecting hairs from a crime scene, it can be interesting for STR analysis to include that part of the tape where the hair root was located.

Success rate experimental data

In the experimental data presented above, 47 of 145 hair roots showed visible nuclei after DAPI staining. 44 of them resulted in a full or partial STR profile, which means that a success rate of 94% could be obtained. Assuming all these hairs would have been found at a crime scene and no screening method would have been applied, all 145 hairs would have been submitted to STR analysis and a success rate of only 30% (44/145) would have been obtained. This shows the effectiveness of this fast screening method using DAPI to select hairs suitable for successful STR analysis.

Forensic cases

The presented fast screening method was applied in 36 forensic cases in which 279 hair roots were stained with DAPI directly on microscope slides (part II). 263 hair roots were quoted negative. Although hair roots without visible nuclei were not selected for STR analysis, 8 of these hair roots were submitted to STR analysis anyway because adherent material was present around the hair root. However, no STR profile could be obtained on these hair roots. All hair roots containing any nuclei (n = 16), were submitted to STR analysis. Results are shown in **Table 4**.

Table 4: STR profiling of hair roots stained with DAPI directly on microscope slides in forensic cases

Number of visible nuclei	Total root N	N roots with STR profile		
		Full profile	Partial profile	No
<20	6	1	2	3
20<n<50	4	2	2	0
>50	6	6	0	0

Similar to the experimental data, full STR profiles could be obtained on the 6 hair roots with more than 50 visible nuclei. Two hair roots containing 20 to 50 nuclei, (one of them collected from an adhesive film), resulted in a full STR profile, while the other 2 resulted in a partial STR profile. As mentioned earlier, minimum 20 nuclei are required to obtain at least partial STR profiles. From the 6 hair roots with less than 20 visible nuclei, 1 resulted in a full STR profile, 2 in a partial STR profile and the other 3 in no profile.

Using the proposed fast screening method, all hair roots containing any nuclei should be submitted to STR analysis. However, one needs to keep in mind that the success rate of STR analysis of hair roots collected from a crime scene could be lower than the observed experimental success rate as adverse environmental condition prior to collection could influence the results.

Conclusion

In conclusion, a fast screening method using DAPI to stain nuclear DNA in hair roots collected at a crime scene can be used to predict STR analysis success. This non-destructive, quick and inexpensive screening method which does not require an incubation time, allows the forensic DNA laboratory to analyze only the most promising hair roots, containing any nuclei. Therefore, judiciary costs can be reduced. DAPI-negative hair roots can result in a STR profile in 4% of the cases, which means that each forensic case has to be evaluated in function of the importance of the evidential value of the found hair.

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CHAPTER 3

Evaluation of a visualization assay for blood on forensic evidence

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Abstract

In forensics, bloodstains on dark fabrics might be invisible for the naked eye. Although several visualization, presumptive and confirmatory blood tests have been developed, all have one or more disadvantages, especially on DNA analysis.

We report the use of a visualization assay that can visually detect blood drops up to 1/20 dilution. In this assay, the fabric is placed between 2 wet filter papers and covered by glass surfaces on both sides. Pressure is applied on the glass surfaces in which bloodstains transfer onto the filter papers through capillary forces. Detected stains can be tested with other more sensitive presumptive blood performed on the filter paper. Even more, DNA analysis can be performed on the transferred bloodstains.

This presented visualization is easy to perform, extremely cheap, requires little hands on time and does not affect bloodstain pattern analysis.

Introduction

One of the first steps in the analysis of forensic evidence is the search for biological material that can subsequently be used for DNA extraction and profiling. Many types of body fluids on forensic evidence, originating from suspect or victim, can be used for DNA profiling, such as blood, semen, saliva, vaginal fluid, urine and sweat [1]. Blood is a common body fluid detected on pieces of evidence found at crime scenes, especially at scenes of violent crimes. Before DNA profiling can be performed, the presence of a potential biological stain, e.g. blood, needs to be visually detected. White light and the visible eye or a low power microscope can be used for a standard examination of an item of clothing. However, when blood is present on a dark background, e.g. a dark fabric, it might be invisible for the naked eye.

Several visualization, presumptive and confirmatory blood tests have been developed and evaluated [2-8]. The potential presence of blood on a dark background can be visualized by an alternate light source such as Polilight [9,10]. Blood does not show significant autofluorescence but has its absorption maximum at 415 nm. As an alternative, infrared (IR) illumination can be used [8] in which an additional light source emitting wavelengths between 800 and 1000 nm, an IR-sensitive camera and image capture software package are necessary to visualize bloodstains. However, this is not standard equipment present in a forensic DNA lab.

Other blood tests can basically be divided into two groups: presumptive blood tests based on the peroxidase activity of hemoglobin (Hb), the oxygen-transport metalloprotein present in red blood cells, and confirmatory blood tests based on immunological testing.

One of the oldest visualization/presumptive tests for blood is the luminol test [4]. Luminol exhibits chemiluminescence when oxidized by Hb [11], is specific for blood and insensitive for other biological fluids. Luminol can, for example, be used to detect latent bloodstains on large surfaces such as a concrete floor at a crime scene where the offender tried to efface blood by thorough cleaning. However, luminol cannot be used for the detection of bloodstains on dark fabrics as DNA degradation can occur [7,12]. Moreover, luminol needs to be applied in a dark room. Similarly, the fluorescein test is based upon oxidation of fluorescin to fluorescein in the presence of Hb and hydrogen peroxide [3,5,6]. Fluorescein fluoresces when exposed to light at 425 - 485 nm (blue light). The necessity of a specific light source and working in the dark can be quite impractical when analyzing forensic evidence [1].

Chemical catalytic presumptive blood tests described are benzidine, tetramethylbenzidine, leucomalachite green and ortho-toludine which show different sensitivities [2,13-15]. Benzidine and

ortho-toludine are potent carcinogens [14,16]. Another presumptive blood test based on the peroxidase activity of Hb, is the Kastle-Meyer test [17,18]. However, these tests are not visualization tests. They only indicate if a visible stain on a fabric is blood or not.

Non-catalytic confirmatory blood tests, e.g. Hexagon OBTI, HemeSelect and ABAcard HemaTrace, are based upon the reaction of antigens of human Hb with anti-human Hb antibodies labeled with a dye and are used to confirm if the blood is human or not. However, primate blood from different species may react with these tests [1,19]. As with the chemical catalytic presumptive tests, they can not be used for the visual detection of bloodstains on dark backgrounds.

In this study, we report the use and validation of a visualization assay for detection of blood, especially on (dark) fabrics. It is based on the transfer of blood, through capillary forces, onto a filter paper in order to locate blood on the fabric. Detection limit and compatibility with other presumptive blood tests were evaluated, as well as the influence of fabric types (natural, synthetic or a combination of both) and weaving pattern on blood detection.

Materials and methods

Samples

Blood samples from a healthy volunteer were collected by venous puncture in EDTA sample tubes.

In a first experiment, the visualization assay was performed on undiluted bloodstains of 20 µl, 10 µl, 5 µl and 1 µl applied on a white and on a dark fabric in order to evaluate the usability of the presented visualization assay for dark fabrics.

Subsequently, the detection limit of the visualization assay was determined. 10, 2, 1 and 0.5 µl of undiluted blood and 20 µl of 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 and 1/500 diluted blood was added in duplicate on a 100% firmly woven white cotton (i.e. fabric used for T-shirts). Moreover, the visualisation test was evaluated in combination with a presumptive blood test (Kastle-Meyer test [17,18], LumiScene [7] and fluorescein test [3,6]). After the visualization assay was performed, fabrics and filter papers were dried in a fume hood upon which the presumptive blood tests were performed on both the blood spot on the fabric and the transferred blood spot on the filter paper. All tests were performed in duplicate at room temperature. The influence of all these tests on DNA profiling was determined.

The visualization assay was repeated on 11 different types of stained and unstained fabrics (both natural and synthetic). All experiments were performed in duplicate at room temperature.

Visualization assay

Two filter papers (VWR International, Radnor, USA) were sprayed with sterile water until the filter was entirely wet. One filter was placed on a clean glass surface. Subsequently the fabric was placed on this filter and covered by the second filter and another clean glass plate. Pressure was applied by placing ~10 kg of weights on top of the second glass plate. During 2 hours, the filter papers were evaluated through the glass every 10 minutes. If red spots were observed on the filter paper, the visualization assay was considered to be positive and indicated the presence of blood. When no spots on the filter paper were observed, the test was considered to be negative.

Kastle-Meyer test

Kastle-Meyer (KM) reagent [17,18] was prepared by dissolving 0.2 g phenolphthalein powder (VWR International) in 10 ml of a 20% sodium hydroxide solution with 2 g mossy zinc (VWR international). After boiling the solution under reflux, 3 ml of the KM reagent was added to 10 ml distilled water and 2 ml 70% ethanol (VWR international). The KM test was performed as described earlier [20]. A piece

of sterile filter paper (Sigma Aldrich, St. Louis, MO, USA) was rubbed gently on a small area of the stain on the fabric. A drop of 70% ethanol was added to the filter paper followed by 1 drop KM solution. Subsequently, a drop of 3% hydrogen peroxide (Sigma Aldrich) was added. An immediate pink color was indicative of blood. Alternatively, the KM test was also performed on the transferred bloodstains on the filter paper of the visualization assay.

LumiScene test

The LumiScene working solution was prepared by adding 5 ml of LumiScene stock solution (LumiScene Field Kit, Loci Forensics B.V., Le Nieuw-Vennep, The Netherlands) to 245 ml Milli-Q water. One activation tablet was added to this solution. After 5 and 10 minutes respectively, this solution was stirred for one minute. The working solution was loaded in the reservoir of a spray system with compressor (HLO 215/25, Fribel NV, Kontich, Belgium). The solution was sprayed on the fabrics or on the filter papers of the visualization assay for 3 seconds. The stains emitted light at 525 nm, which is visible for the naked eye [7].

Fluorescein test

The fluorescein stock solution was prepared by mixing 1.6 g NaOH (Merck, Darmstadt, Germany), 2 g zinc powder (Panreac, Barcelona, Spain), and 0.16 g fluorescein ($C_{20}H_{12}O_5$, Sigma-Aldrich) with 20 ml of distilled water. After vigorous shaking, the solution was left for one hour to let the zinc powder settle. Meanwhile, 99 ml of oxidant was prepared by mixing 5 ml H_2O_2 (Merck, Darmstadt, Germany) with 94 ml of distilled water. The fluorescein working solution was prepared by adding 1 ml of the stock solution to the oxidant, carefully avoiding the addition of zinc powder. The working solution was then loaded into an Ecospray system (Carl Roth, Lauterbourg, France). The solution was sprayed on the fabrics or on the filter papers of the visualization assay for 3 seconds, while the stains were exposed to light of a portable xenon-lamp with a filter for emission at 450 nm (Crime Scene lamp SL-450, Heerbrugg, Switserland). The stains emitted light in the 500-590 nm range, which could be viewed using yellow forensic goggles [3,6].

DNA extraction

DNA was extracted from all bloodstains applied on the 100% white cotton fabrics and from the transferred bloodstains on the filter paper using a slightly modified Chelex® extraction method as described earlier [21]. In a first step, bloodstains were cut from the fabric and from the filter paper with a sterile scalpel. These stains were incubated in 1 ml sterile water for 30 minutes at room temperature. After incubation, the fabrics/filter papers were removed and samples were centrifuged at 14000 rpm for 5 minutes. Subsequently, supernatant was removed and the pellet was

resuspended in 200µl 5% Chelex® solution (Chelex®100 resin, Bio-Rad, Hercules, CA, USA). After incubation at 56°C for 30 minutes, the samples were put in boiling water for 8 minutes. After centrifugation at 14000 rpm for 3 minutes, 30 µl of the supernatant was used for PCR.

DNA amplification and capillary electrophoresis

All samples were amplified using an in house developed multiplex of 14 short tandem repeat (STR) loci (D3S1358, TH01, D21S11, D18S51, vWA, D8S1179, TPOX, FGA, D5S818, D13S17, SE33, CD-4, D7S820 and D16S539) and the amelogenin locus [21]. Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany) or Life Technologies (Carlsbad, CA, United States of America). Each reaction mix, with an end volume of 50 µl, contained 16.55 µM primer mix, 1x PCR buffer (Qiagen, Venlo, The Netherlands), 0.5 mM $MgCl_2$ (Qiagen), 200 µM dNTP (Applied Biosystems), 0.4 µg/µl albumin (Sigma Aldrich), 5 U Hotstar Taq polymerase (Qiagen) and 30 µl DNA extract. The samples were amplified on an Applied Biosystems GeneAmp 9700 60-well thermal cycler. Amplification parameters were: preincubation at 95°C for 15 minutes, followed by 34 cycles of denaturation for 60 seconds at 94°C, annealing for 60 seconds at 59°C and extension for 80 seconds at 72°C. This was followed by a final elongation step of 25 minutes at 72°C. At the end of the PCR reaction, the temperature was kept at 4°C. After PCR, amplified fragments were separated and analyzed by capillary electrophoresis using an ABI PRISM® 3500xL Genetic Analyzer equipped with Genemapper ID-X 1.2 software (Applied Biosystems). Peak height minimum thresholds were set at 100 relative fluorescence units (RFU). Each DNA profile of an analyzed blood spot was compared to the DNA profile of the donor of the bloodstain.

Results and discussion

Detection limit of the visualization assay

The presented visualization test is especially meant for the visualization of bloodstains on dark fabrics. Therefore, undiluted bloodstains of 20 µl, 10 µl, 5 µl and 1 µl were applied on a white (**Figure 1A**) and on a dark fabric (**Figure 1C**). Bloodstains on the dark fabric were invisible for the naked eye. After 30 minutes incubation, bloodstains on both fabrics were made visible on the filter paper of the visualization assay (**Figure 1B** and **1D** respectively).

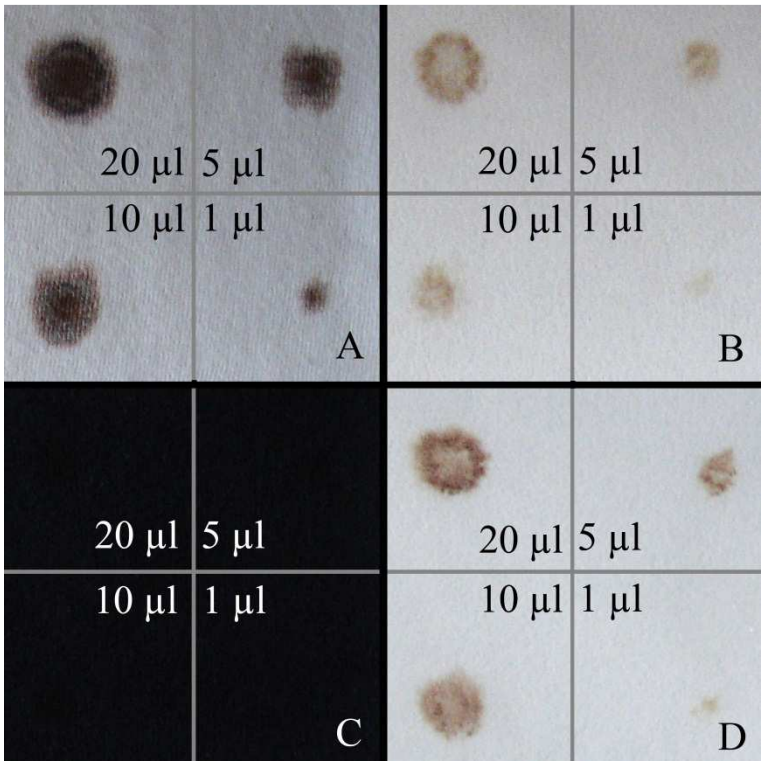

Figure 1: Undiluted blood stains of 20µl, 10µl, 5 µl and 1 µl on a white fabric (A) and on a dark fabric (C), visualized by the visualization assay (B and D respectively). Bloodstain pattern (A) remains unaffected with the visualization assay (B).

Subsequently, the detection limit of the visualization assay was determined by applying 10, 2, 1 and 0.5 µl of undiluted blood and 20 µl of 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 and 1/500 diluted blood on a 100% firmly woven white cotton. The visualization assay was positive for the undiluted blood spots and blood spots diluted up to 1/20. As most bloodstains on forensic evidence are undiluted and in many cases only present in very small amounts, the assay can easily be used to visualize bloodstains on dark fabrics, e.g. clothes recovered from a crime scene.

Visualization and interpretation of latent bloodstains is an essential part of bloodstain pattern analysis (BPA), which is an important part of the investigation and crime scene reconstruction [22].

The bloodstain pattern remains unaffected with the presented visualization assay, which is clearly shown in **Figure 1A** and **1B**. Therefore, the visualization assay can be used to perform a BPA on a dark fabric by evaluating the transferred bloodstains on the filter paper (**Figure 1D**).

Combination of the visualization assay with other presumptive blood tests and subsequent DNA profiling

In a first experiment, the visualization assay was performed on a bloodstain on the fabric followed by a presumptive blood test on the same blood spot on the fabric. Although blood spots diluted more than 1/20 did not always test positive with the visualization assay, all visible blood spots on the fabric, diluted up to 1/500, tested positive for the presumptive blood tests, even if the visualization assay was performed earlier on the same spot on the fabric. Secondly, a presumptive blood test was also performed on the dried transferred blood spot on the filter paper. These blood spots on the filter paper tested positive for the presumptive blood tests. Even more, bloodstains hardly visible on the white fabric and not visually detected on the filter paper (blood diluted 1/500), tested positive with the KM test which was performed on the filter paper. In real forensic cases however, the KM test cannot be performed on invisible stains on the filter paper. Hence, we recommend spraying, for example, LumiScene on the filter paper in order to relocate bloodstains on the piece of evidence to be analysed. The advantage of this technique is that no DNA degradation of the stain on the fabric will occur.

Regardless of the fact that the applied stain on the fabric was positive by the visualization assay, the stain on the fabric was subjected to DNA extraction and DNA profiling. All stains on the fabric generated a full DNA profile. Transferred bloodstains on the filter paper of undiluted blood spots and blood spots diluted 1/2, 1/5, 1/10 and 1/20, were submitted to DNA analysis. Full DNA profiles could be obtained on the filter paper of the undiluted bloodstains and bloodstains diluted 1/2. Therefore, there is no need to cut the bloodstain from the piece of fabric to obtain a DNA profile, as the transferred bloodstain on the filter paper can be used for DNA profiling. If no (full) DNA profile can be obtained from the filter paper, the stain itself should be submitted to DNA analysis.

Our data show that the visualization assay can be combined with a presumptive blood test without influencing the potential of obtaining a DNA profile from the bloodstain. The visualization assay has to be performed first since it is a simple, easy to perform and, most importantly, inexpensive technique to visualize latent bloodstains on dark fabrics. If a positive result with the visualization assay is obtained, it should be confirmed by a presumptive blood test applied on the filter paper prior to DNA profiling. A confirmatory blood test, e.g. Hexagon OBTI test, could be performed on the bloodstain to confirm if the blood is human or not [19]. If the visualization assay is negative, this

means that either no blood is present on the fabric or that the blood is diluted more than what is visually detectable with the visualization assay. However, the most important bloodstains on the fabric useful for DNA analysis, will be made visible with the presented assay. In case the visualization assay is negative, one might consider to perform a more sensitive and more expensive presumptive blood test, for example LumiScene, on the filter paper or on the fabric. Detection of blood which is diluted more than 1/500 is interesting for forensic scientists to prove the presence of latent blood. This is especially the case when the offender tried to efface a bloodstain by thorough cleaning. However, it is less relevant for human identification by DNA profiling as these stains will theoretically not lead to useful DNA profiles.

Influence of the type of fabric

To investigate whether the type of fabric has an influence on the sensitivity of the visualization assay, bloodstains and blood dilutions were applied on 11 different stained and unstained fabrics of natural and (semi-)synthetic origin. Results are shown in **Table 1**. Undiluted blood drops of 10 µl and 2 µl on all fabric types were visually detected on the filter paper. In most cases, the visualization assay was positive for smaller drops of undiluted blood. 95.5% of 1 µl and 86.4% of 0.5 µl blood drops were visually detected on the filter paper. No clear trends were observed between different types of fabrics for undiluted blood.

The type of fabric (synthetic, natural or a combination of both) on which the (diluted) blood is present, has some influence on the potential to visualize it with the presented visualization assay. With the visualization assay, 20 µl of 1/2 diluted blood was visually detected on all fabrics, while 1/500 diluted blood was not visually detected on the filter paper on any of the fabrics. Blood dilutions in between those two extremes were visually detected on the filter paper as follows: 1/5: 90.9%; 1/10: 72.7%; 1/20: 59.1%; 1/50: 22.7% and 1/100: 13.6%. Comparison of blood detection on natural fabrics and (semi-)synthetic fabrics showed slightly better results for natural fabrics. The 1/20 dilution, for example, was visually detected on the filter paper on 83.3% of the natural fabrics, visually undetectable on the synthetic fabrics and only visually detectable on 50.0% of the semi-synthetic fabrics. Some difference between loosely and firmly woven fabrics was observed. On loosely woven fabrics, somewhat more positive results with the visualization assay could be obtained compared to (very) firmly woven fabrics. 1/20 diluted blood was visually detected on the filter paper on all loosely woven fabrics in contrast to 35.7% of the (very) firmly woven fabrics. Moreover, 1/50 and 1/100 diluted blood were visually detected with the assay on 85.3% and 50.0% of the loosely woven fabrics, while this was impossible on the (very) firmly woven fabrics. However, on very firmly woven fabrics, a more sensitive presumptive blood test can be performed.

Table 1: Evaluation of the visualization assay on 11 different types of fabrics, stained or unstained, each test performed in duplicate.

Fabric	1	2	3	4	5	6	7	8	9	10	11
Type	Natural	Natural	Natural	Natural	Natural	Natural	Synthetic	Synthetic	Semi-synthetic	Semi-synthetic	Semi-synthetic
Composition	100% cotton	100% cotton	100% cotton	100% wool	100% wool	100% leather	100% acetate	90% polyacryl-amide, 10% elastane	65% poly-ethylene, 35% cotton	65% poly-ethylene, 35% cotton	80% wool, 20% nylon
Weaving	Firmly	Loosely	Very firmly	Loosely	Firmly	/	Firmly	Firmly	Firmly	Firmly	Loosely
Garment	T-shirt	T-shirt	Sheet	Jacket collar	Jacket	Jacket	T-shirt	T-shirt	T-shirt	T-shirt	Jumper
Color	Unstained	Dark grey	Unstained	Black	Black	Dark brown	Dark grey	Dark red	Dark grey	Red	Dark green
Blood spot											
10 µl	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
2 µl	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
1 µl	+ / +	+ / +	+ / +	+ / +	+ / -	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
0.5 µl	+ / +	+ / +	+ / -	+ / -	+ / +	+ / +	+ / +	+ / -	+ / +	+ / +	+ / +
20 µl 1/2	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
20 µl 1/5	+ / +	+ / +	+ / +	+ / +	+ / +	+ / +	+ / -	+ / -	+ / +	+ / +	+ / +
20 µl 1/10	+ / +	+ / +	+ / -	+ / +	+ / +	+ / +	+ / +	+ / -	- / -	- / -	+ / +
20 µl 1/20	+ / +	+ / +	- / -	+ / +	+ / +	+ / +	- / -	- / -	- / -	+ / -	+ / +
20 µl 1/50	- /-	+ / +	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / +
20 µl 1/100	- / -	+ / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	+ / +
20 µl 1/500	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -

+ / + Both blood spots on the fabric tested positive

+ / - Only one of the two blood spots tested positive

- / - Both blood spots on the fabric tested negative

Conclusions

Blood is a common type of body fluid found at crime scenes, particularly at scenes of violent crimes. On dark fabrics, it can be quite challenging to visually detect blood with the naked eye, hence visualization aids, such as Polilight, fluorescein test and luminol test, have been developed. All of these have one or more disadvantages, such as a potential negative impact on DNA quality, impracticality (e.g. necessity of fluorescent illumination or darkened room) and/or high cost. In an attempt to overcome these disadvantages, a visualization assay for the detection of blood on dark fabrics was optimized and validated in this study.

This visualization assay, based on migration of blood to a filter paper through capillary forces, shows no negative influence on DNA quality of the stain. Moreover, presumptive blood tests to confirm the presence of blood and DNA profiling can be performed on the transferred bloodstains on the filter paper. Evaluation of the presence of blood can be performed after 30 minutes incubation, while the glass plate is still on top of the fabric, protecting it from potential contamination. It is easy to perform, requires little hands on time and is extremely cheap because only filter paper, water and two glass plates are needed. Moreover, the presented visualization assay does not disturb bloodstain pattern, which is an important part of the investigation and crime scene reconstruction.

Although the visualization assay is less sensitive for diluted blood, this can be overcome by combining it with a standard presumptive blood test. Overall, it can be concluded that the presented visualization assay is easily applicable and is a valuable technique for visualization of blood on dark fabrics.

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PART IV

Summary and final discussion

During this PhD thesis, several visualization techniques have been optimized to study clinical and forensic samples. The use of a microscope is of utmost importance to study these samples at the cellular and subcellular level. However, most fixed cells are hardly visible through a light microscope without staining techniques. Therefore, several staining techniques have been optimized and validated throughout this thesis in order to visualize male fetal microchimeric cells in blood of patients with an autoimmune thyroid disease and to count nuclei in hair roots in the context of forensic investigation. As blood is a common type of body fluid found at crime scenes but hardly visible on dark backgrounds, a method to visualize latent bloodstains on dark fabrics has been optimized (**Part I**).

In a first part of this thesis, the potential role of fetal microchimeric cells in two common autoimmune thyroid diseases (AITDs), Hashimoto's thyroiditis (HT) and Graves' disease (GD), was investigated (**Part II**). Fetal microchimerism refers to the presence of fetal cells in maternal blood and tissues during and after pregnancy, and is a well-known phenomenon in healthy persons as well as persons with a (autoimmune) disease (**Part II, Chapter 1**). HT is the most common cause of hypothyroidism, while GD the major cause of hyperthyroidism (**Part II, Chapter 3**).

To identify male fetal microchimeric cells in women with a previous male pregnancy, two techniques have been most widely used: PCR targeting sequences specific to Y chromosome genes (e.g. *DYS14* and *SRY*) and Fluorescence *in situ* Hybridization (FISH) using X and Y chromosome specific probes. While PCR only indicates the presence of fetal cells and estimates the amount of fetal microchimeric cells, FISH gives an exact number. Both techniques have a different sensitivity. With PCR, a single male cell can be detected within a background of 100.000 female cells, compared to 1 male cell within 2 million female cells with FISH. Moreover, FISH can be used to locate fetal cells in a tissue section and at the same time, these cells can be characterized using fluorescently labelled antibodies. DAPI is used to stain all nuclei of the sample. Because of the higher sensitivity, FISH has been used in our study to identify male fetal microchimeric cells in blood of patients with HT or GD (**Part II**, **Chapter 4**). A disadvantage of FISH is the labour-intensive analysis of the results and the possible occurrence of false positive results. However, applying a second round of FISH with another Y chromosome probe decreases the number of false positive results. The search for desired cells on a slide can often be time consuming, especially if the cells of interest are rare. Image analysis software modules can be very helpful for the automatic detection of target cells, e.g. spermatozoa stained with Sperm HY-LITER™ and male buccal cells in an overwhelming amount of female cells [1,2]. Software can also be used to automatically count cell nuclei on a slide [3]. As male fetal microchimeric cells in maternal blood are rare, an image processing AxioVision Commander module

based on the detection of the Y chromosome spots, was used for the automatic detection of these cells. The cells automatically detected with the software, were relocated on the slide, and visual inspection of the cells was performed to make sure the detected cells were not false positives.

More male fetal microchimeric cells were detected in blood of patients with GD or HT compared to healthy controls. At the same time, a significant difference in number of male fetal cells was observed between both groups of patients. Therefore, fetal microchimeric cells were characterized by enriching B, $CD4^+$ T and $CD8^+$ T cells prior to FISH. Our study focused on B and T cells as these cell types are more likely to initiate or be involved in immune response. In patients with HT, mainly male fetal $CD8^+$ cytotoxic T cells were found. One might speculate that these fetal T cells could cause cell death leading to hypothyroidism. In patients with GD, the majority of male fetal cells was found in the B cell fraction. These fetal B cells could possibly be activated by fetal $CD4^+$ T cells, which were also detected in blood of these patients. One might speculate that activation of fetal thyroid-specific $CD4^+$ T cells leads to recruitment of autoreactive B cells. These B cells could secrete TSHR stimulating antibodies leading to hyperthyroidism. Although our results show a clear association of fetal microchimerism with AITD and indicate a potential harmful effect of these fetal cells in the pathogenesis of HT and GD, we have to keep in mind that results of this study are based on a limited number of patient samples and therefore should be confirmed in a larger data set. Even more, fetal microchimeric cells have to be characterized more in depth to define 'the other cell types'.

Several technical and clinical issues have to be considered if data on fetal microchimerism are compared. Differences in experimental design, sensitivity and specificity of the detection techniques (as mentioned above) could influence the results. Additionally, some reports lack data concerning pregnancy history which is important if fetal microchimerism is studied. Terminations of pregnancy for example, are associated with an elevated number of fetal microchimeric cells [4,5]. Moreover, alternative sources of microchimerism, natural or iatrogenic, should be taken into account as a recent publication stated that we are all born as microchimera [6]. Therefore, microchimeric cells should be isolated from the maternal blood in order to determine the possible origin of these cells. These microchimeric cells could have a fetal, maternal or grandmaternal origin, or could be originating from a blood transfusion. A possible technique to determine the origin, is to perform HLA typing on the isolated microchimeric cells and to compare the HLA type with their children, mother and other possible donors of the microchimeric cells. However, more research needs to be done on obtaining a pure population of microchimeric cells.

These above mentioned concerns aside, fetal microchimerism has been shown to be more common in thyroid glands of patients with AITD compared to controls [7-9]. In contrast to thyroid tissue where

fetal cells could be detected in only 50% of patients with an AITD, fetal cells were detected in blood of all patients in our study. However, it is possible that in patients who appear to be negative for male fetal microchimerism, fetal cells could not be detected by the methods used due to their lack of sensitivity. Moreover, female instead of male fetal microchimeric cells could be present in blood and/or tissues of these patients. Up to now, female fetal microchimerism can only be identified based on differences in HLA between mother and fetus and is more difficult to study because the system requires multiple detection probes. During this PhD thesis, we have tried to optimize a HLA-based detection technique. However, results were disappointing because of high background staining which could not be reduced despite several attempts to optimize the staining. Therefore, no software could be used for the automated detection of these rare cells. Optimizing staining techniques and development of more sophisticated detection techniques is mandatory to be able to detect female fetal microchimeric cells in blood and tissues of patients with an autoimmune (thyroid) disease. Once achieved, female microchimerism could also be studied in men with an autoimmune disease.

In thyroid glands where the immune reaction is taking place, only the presence of fetal microchimeric cells has been proven [7-9]. To elucidate the potential role of fetal microchimeric cells in AITD, fetal microchimeric cells in the thyroid gland have to be characterized. Currently, preliminary data are obtained from thyroid glands of patients with GD. Although fetal cells were detected in their thyroid glands, characterization studies did not (yet) confirm the presence of fetal T or B cells. Further research to determine the fetal cell type in the thyroid gland is mandatory to assign a potential harmful effect of these cells in autoimmune thyroid diseases. If fetal cells detected in the thyroid gland do not turn out to be fetal B or T cells, other stainings need to be optimized and performed to determine the fetal cell type.

To conclude the first part of the PhD thesis, a review discussing hypotheses about the potential role of fetal microchimeric cells in autoimmune (thyroid) diseases has been described (**Part II, Chapter 5**). Fetal cells could have harmful, beneficial or innocent effects for the thyroid gland. In a harmful way, fetal cells could cause autoimmune disease by initiating a graft-versus-host reaction, or the maternal host could initiate a host-versus-graft reaction against these fetal cells. Despite the fact that male fetal cells were only characterized in blood in a limited number of patient samples in our study, the presence of fetal $CD8^+$ T cells in HT and fetal $CD4^+$ T and B cells in GD was clearly shown. These results provide support for the hypothesis that fetal microchimeric cells could have a harmful effect in the thyroid gland in which fetal cells could initiate a graft-versus-host reaction in the mother. Our data indicate the value and need for further research in this field. A beneficial effect of fetal microchimeric

cells in which fetal cells could offer help in tissue repair, has mainly been described in breast and cervical cancer. A third hypothesis states that microchimeric cells are innocent bystanders in the process of autoimmunity as microchimeric cells have also been detected in healthy women. In our opinion however, the harmful, beneficial or innocent effect of fetal microchimerism depends on several factors including the type of fetal cells acquired, tissue environment and type of malignancy. More importantly, it seems that HLA similarities between fetal and maternal cells have the potential to affect the balance of beneficial versus harmful consequences for the mother. The more fetal microchimeric cells show HLA similarities with maternal cells, the less likely they are recognized by the maternal immune system and the more likely they have the potential to start a graft-versus-host reaction once they have been activated by yet undetermined mechanisms. Further investigation to confirm this hypothesis is required.

Insights in the mechanism by which fetal microchimeric cells have potential harmful effects on autoimmune thyroid diseases, could lead to the development of a specific therapy targeting the fetal immune cells. In Graves' disease for example, fetal B cells, possibly responsible for production of anti-thyroid stimulating antibodies causing hyperthyroidism, could be eliminated by Rituximab, a monoclonal antibody against CD20 which is primarily found on the surface of B cells. Rituximab is already used for the treatment of the autoimmune disease rheumatoid arthritis [10]. A side effect of this depletion therapy however, is that also non-autoreactive B cells are depleted. Therefore, it is important to search for a therapy that specifically targets the fetal cells. The first step however, is to get more insights in the mechanism on how fetal microchimeric immune cells could start an autoimmune response in the mother.

In a second part of this thesis, two visualization techniques have been optimized for their use in forensic applications (**Part III**). In our lab, several (fluorescent) staining techniques have been developed to analyze forensic samples. In cases of sexual assault for example, FISH can be used to discriminate male cells of the perpetrator from female cells of the victim. Subsequently, male cells can be isolated using laser pressure catapulting in order to obtain a single DNA profile of the perpetrator instead of mixed DNA profiles, which are more difficult to interpret [2].

The fluorescent stain DAPI has also its application in forensic research. We evaluated the use of DAPI to stain nuclear DNA in hair roots to predict their DNA analysis success rate (**Part III, Chapter 2)**. Human hairs are frequently recovered as forensic evidence on crime scenes as humans shed around 150 hairs daily. These hairs can be microscopically compared to reference hairs of the putative offender. However, the discriminative power of microscopic analysis of hairs is relatively small. Therefore, Short Tandem Repeat (STR) analysis of the hair root should be performed to identify the

donor of the hair. The success rate of STR profiling of hair roots however, is quite low and negative results of hair analysis are frequently reported. To increase the success rate, a screening method prior to DNA analysis should be performed to select hairs containing nuclear DNA. Nuclear DNA in hair roots can be stained with haematoxylin [11]. However, haematoxylin is known to reduce DNA yield and is therefore not recommended if these hair roots are submitted to DNA analysis afterwards. Another technique to visualize nuclear DNA, is labeling *in situ* [12]. As mentioned above, labeling *in situ* is very labor-intensive. Therefore, the use of DAPI to stain nuclear DNA in hair roots overnight has been proposed [13]. The current Belgium DNA law demands a forensic DNA report within a month. Therefore, protocols should be kept as short as possible. The aim of this study was to improve the staining technique for use in forensic DNA laboratories. We were able to reduce the incubation time from overnight to a direct staining and visualization of the hair root under the fluorescence microscope. DNA analysis of hair roots counting any nuclei, resulted in a success rate of 94%. Without this screening method, a success rate of only 30% would have been achieved, which indicates the value of performing our fast screening method prior to DNA analysis. If hairs are collected from adhesive films, it can be interesting for DNA analysis to include that part of the tape where the hair root was located as loss of nuclei after removing the hair root from the adhesive film was observed. In an optimal situation, hair roots without visible nuclei should not be selected for DNA analysis since no profiles could be obtained in 96% of the analyzed samples. However, in 4% of these cases, a full or partial DNA profile could be obtained. This could possibly be due to adhering material around the hair root. Therefore, results of DAPI staining should always be considered in function of the importance of the evidential value of the found hair. If the hair is the only biological evidence in the forensic case and the staining is considered to be negative, one might consider to submit the hair to STR analysis anyway. Multiple hair roots having the same characteristics can be pooled for STR analysis, possibly increasing the chance of obtaining a DNA profile. If still no STR profile could be obtained, one might consider to submit the remainder of the hair to mitochondrial DNA analysis. However, mitochondrial DNA is the same in the maternal lineage and is therefore less discriminative to autosomal STR analysis.

This screening method has now been implemented in our forensic DNA laboratory. Similar results were obtained on hair roots selected from 36 forensic cases. Without this screening method, 279 hair roots would have been submitted to DNA analysis, which is associated with a high judiciary cost. We only selected 16 hair roots for DNA analysis based on the presence of nuclei. DNA profiles could be obtained in 81% of these cases. In the remainder 19% however, less than 20 nuclei, which are required to obtain at least partial STR profiles, were detected. Moreover, we have to keep in mind that adverse environmental conditions prior to collection of hairs from crime scenes could influence

the results. These data indicate the value of this screening method in order to reduce judiciary costs. We recommend all forensic DNA laboratories to use this fast screening method for the selection of hairs suitable for DNA analysis.

One of the first steps in the analysis of forensic evidence is the search for biological material that can subsequently be used for DNA extraction and profiling. Blood is a common body fluid detected on pieces of evidence found at crime scenes, especially at scenes of violent crimes. However, bloodstains on dark backgrounds, e.g. dark fabrics, are hard to see with the naked eye. Several visualization techniques such as Polilight, infrared detection, luminol based detection and fluorescein, have been suggested to locate blood spots on dark backgrounds. However, all are associated with one or more disadvantages, including the need to work in a dark room, the need for alternative light sources, the occurrence of false positive or false negative results, a high cost or negative influence on DNA quality. Therefore, the aim was to develop a simple, inexpensive visualization assay without the need of an alternative light source (**Part III, Chapter 3**). The presented visualization assay is based on the transfer of blood onto a filter paper through capillary forces in order to locate bloodstains on a dark piece of fabric. Small quantities of undiluted bloodstains on several kinds of fabrics can easily be detected with the presented assay. This is of utmost importance since most bloodstains found on forensic evidence are undiluted and present in small amounts. In case of diluted bloodstains, slightly better results were obtained for loosely woven natural fabrics compared to firmly woven synthetic fabrics. As this visualization assay is only a visualization aid for detecting bloodstains on dark fabrics, a positive result should always be confirmed by a presumptive blood test, e.g. Kastle-Meyer test.

This presumptive blood test can be applied on the detected bloodstain on the fabric, but it can also be applied directly on the filter paper of the visualization assay, which is a major advantage of the presented technique. The human origin of the bloodstain can be confirmed using the Hexagon OBTI test. If no blood spots are visualized on the filter paper, this means that either no blood is present on the fabric or that the blood is diluted more than what is visually detectable with the visualization assay. In that case, one might consider to perform more sensitive and more expensive presumptive blood tests on several locations of the fabric. However, the most important bloodstains useful for DNA analysis will be detected with the visualization assay. Besides the low cost and the easiness to perform the assay, two important advantages for application in a routine forensic DNA laboratory, DNA analysis can be performed directly on the transferred bloodstains on the filter paper without the need to cut bloodstains from a piece of evidence. In case no DNA profile could be obtained from the transferred bloodstain on the filter paper, the stain on the fabric itself must be used for DNA analysis.

This visualization assay prior to other presumptive blood tests to detect latent blood spots on dark fabrics, is highly recommended for forensic laboratories as it is easy to perform and extremely cheap. Moreover, bloodstain pattern analysis, an important part of crime scene reconstruction, can be performed on the filter paper itself. As from now, the visualization assay will be performed in our DNA laboratory if bloodstains on dark fabrics have to be visualized. However, more research has to be done on mixtures of blood and other biological substances such as saliva and vomit, semen and menstrual blood, in order to determine the influence of these substances on the ability to visualize bloodstains and to perform DNA analysis on the transferred bloodstains.

During this PhD thesis, several visualization techniques were optimized and validated in order to analyze clinical and forensic samples. However, the continuous development of new visualization techniques is necessary to analyze these samples more easily.

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PART V

Samenvatting en finale discussie

Tijdens deze doctoraatsthesis werden verschillende visualisatietechnieken geoptimaliseerd om klinische en forensische stalen te bestuderen. Het gebruik van een microscoop is belangrijk om deze stalen op cellulair en subcellulair niveau te bestuderen. De meeste gefixeerde cellen zijn echter moeilijk te zien doorheen een lichtmicroscoop zonder kleuringstechnieken. In het kader van deze thesis werden verschillende kleuringen geoptimaliseerd en gevalideerd om mannelijke foetale microchimere cellen in bloed van patiëntes met een auto-immune schildklieraandoening te detecteren en om, in het kader van forensisch onderzoek, nuclei in haarwortels te visualiseren. Aangezien bloed een vaak voorkomend type van lichaamsvloeistof is die teruggevonden wordt op een plaats van misdrijf, maar moeilijk zichtbaar is op donkere achtergronden, werd een methode geoptimaliseerd om bloedvlekken op donkere stoffen zichtbaar te maken (**Deel I**).

In een eerste deel van deze thesis werd de potentiële rol van foetale microchimere cellen in twee auto-immune schildklieraandoeningen (AITD) onderzocht, namelijk de ziekte van Hashimoto (HT) en de ziekte van Graves (GD) (**Deel II**). Foetale microchimerie verwijst naar de aanwezigheid van foetale cellen in maternaal bloed en maternale weefsels tijdens en na de zwangerschap, en is een algemeen gekend fenomeen in zowel gezonde personen als personen met een (auto-immune) aandoening (**Deel II, Hoofdstuk 1**). HT is de meest voorkomende oorzaak van hypothyroïdie, terwijl GD de belangrijkste oorzaak van hyperthyroïdie is (**Deel II, Hoofdstuk 3**).

Om mannelijke foetale microchimerie cellen in vrouwen die eerder een zoon hebben gebaard te identificeren, worden er voornamelijk twee technieken gebruikt: PCR met amplificatie van Y chromosomale specifieke sequenties (bv. *DYS14* en *SRY*), en Fluorescentie *in situ* hybridisatie (FISH) met specifieke X en Y chromosomale probes. Met PCR kan enkel de aanwezigheid van foetale cellen aangetoond worden en een schatting gegeven worden van het aantal foetale cellen. Met FISH daarentegen, kan het aantal foetale cellen exact bepaald worden. Bovendien hebben beide technieken een verschillende sensitiviteit. Eén enkele mannelijke cel op 100.000 vrouwelijke cellen kan gedetecteerd worden met PCR, terwijl dit met FISH 1 mannelijke cel op 2 miljoen vrouwelijke cellen is. Tegelijkertijd kunnen foetale cellen gelokaliseerd en gekarakteriseerd worden in een weefselsectie door gebruik te maken van FISH in combinatie met fluorescent gelabelde antilichamen. DAPI wordt gebruikt om alle celkernen in een staal aan te kleuren. Omwille van de hogere sensitiviteit, werd FISH ook in onze studie toegepast om mannelijke foetale cellen in bloed van patiëntes met HT of GD te identificeren (**Deel II, Hoofdstuk 4**). Een nadeel van FISH is de arbeidsintensieve analyse van de resultaten en het voorkomen van vals-positieve resultaten. Door echter nogmaals FISH toe te passen maar nu met een andere Y chromosomale gelabelde probe, vermindert de kans op vals-positieve resultaten. De zoektocht naar de gewenste cellen op een slide

kan vaak tijdrovend zijn, vooral indien de cellen van interesse zeer zeldzaam zijn. Software modules voor beeldverwerking kunnen zeer handig zijn om de cellen van interesse automatisch te detecteren, bv. spermatozoa gekleurd met Sperm HY-LITER™ en mannelijke buccale cellen in een overgrote meerderheid van vrouwelijke cellen [1,2]. Bovendien kan bepaalde software ook gebruikt worden om automatisch het aantal celkernen op een slide te tellen [3]. Aangezien het aantal mannelijke microchimere cellen in maternaal bloed zeldzaam is, werd een beeldverwerkingsmodule van AxioVision gebruikt voor de automatische detectie van deze cellen. Het script is gebaseerd op de detectie van Y chromosomale spots. De automatisch gedetecteerde cellen werden opnieuw gelokaliseerd op de slide en werden onderworpen aan een visuele controle om er zeker van te zijn dat de gedetecteerde signalen geen vals positieve signalen waren.

In vergelijking met gezonde controles werden meer mannelijke foetale cellen gedetecteerd in bloed van patiëntes met GD of HT. Tegelijkertijd werd een significant verschil in aantal mannelijke foetale cellen geobserveerd tussen beide groepen van patiëntes. Daarom werden, voorafgaand aan FISH, foetale cellen gekarakteriseerd door het aanrijken van B, $CD4^+$ T en $CD8^+$ T cellen. De focus van onze studie lag op detectie van foetale B en T cellen omdat deze celtypes meer waarschijnlijk een immuunreactie initiëren of daarin betrokken zijn. In patiëntes met HT werden voornamelijk mannelijke foetale $CD8^+$ cytotoxische T cellen gevonden. Er kan gespeculeerd worden dat deze foetale T cellen celdood kunnen veroorzaken, en uiteindelijk hypothyroïdie. In patiëntes met GD werden voornamelijk mannelijke foetale B cellen aangetroffen. Deze foetale B cellen zouden vermoedelijk kunnen geactiveerd worden door foetale $CD4^+$ T cellen, eveneens gedetecteerd in het bloed van deze patiëntes. We kunnen speculeren dat activatie van foetale schildklier-specifieke $CD4^+$ T cellen aanleiding geeft tot het rekruteren van autoreactive B cellen. Deze B cellen zouden dan TSHR-stimulerende antilichamen kunnen secreteren die aanleiding geven tot hyperthyroïdie. Hoewel onze resultaten een duidelijk associatie van foetale microchimerie met AITD en een potentieel schadelijk effect van deze cellen in de pathogenese van HT en GD aantonen, moeten we in het achterhoofd houden dat de resultaten van deze studie gebaseerd zijn op een beperkt aantal patiënten stalen en dat de resultaten bijgevolg moeten bevestigd worden in een grotere dataset. Bovendien moeten ‘de overige celtypes’ nog beter gekarakteriseerd worden.

Indien data over foetale microchimerie vergeleken worden, moeten verschillende technische en klinische aspecten in overweging genomen worden. Verschillen in experimentele opzet, sensitiviteit en specificiteit van de detectietechnieken (zoals eerder vermeld) kunnen een invloed hebben op de resultaten. Bovendien missen sommige reporten data over de zwangerschapshistoriek, wat belangrijk is als foetale microchimerie bestudeerd wordt. Beëindiging van de zwangerschap

bijvoorbeeld, wordt geassocieerd met een verhoogd aantal foetale microchimere cellen [4,5]. Bovendien moeten alternatieve, natuurlijke of iatrogene, bronnen van microchimerie in rekening gebracht worden aangezien in een recente publicatie wordt gesteld dat we allemaal geboren worden als microchimera [6]. Het is daarom van belang om de microchimere cellen te isoleren uit maternaal bloed om de mogelijke bron van deze cellen te achterhalen. Deze microchimere cellen kunnen een foetale of maternale oorsprong hebben, of kunnen zelfs afkomstig zijn van de grootmoeder. Bovendien kunnen deze cellen ook verkregen zijn via een bloedtransfusie. Een mogelijke techniek om de oorsprong van deze cellen te bepalen, is via HLA typering om vervolgens het HLA type van deze microchimere cellen te vergelijken met het HLA type van hun kinderen, moeder en andere mogelijke donoren van de microchimere cellen. Meer onderzoek is echter in eerste instantie nodig om een pure populatie van microchimere cellen te bekomen voor HLA typering.

Deze bedenkingen terzijde, werden er meer foetale microchimere cellen aangetoond in schildklieren van patiëntes met AITD in vergelijking met controles [7-9]. In tegenstelling tot schildklierweefsel waar foetale cellen slechts gedetecteerd werden in 50% van de patiëntes met AITD, werden foetale cellen aangetoond in het bloed van alle patiëntes in onze studie. Het is echter mogelijk dat in patiëntes die geen mannelijke foetale microchimere cellen blijken te bezitten, foetale cellen niet gedetecteerd werden door het gebrek aan een hoge sensitiviteit van de gebruikte detectietechniek. Bovendien kunnen vrouwelijke in plaats van mannelijke foetale microchimere cellen aanwezig zijn in bloed en/of weefsels van deze patiëntes. Tot nu toe kunnen vrouwelijke foetale microchimere cellen enkel bestudeerd worden aan de hand van HLA verschillen tussen moeder en foetus. Dit is moeilijker te bestuderen omdat meerdere detectieprobes noodzakelijk zijn. In de loop van deze thesis werd getracht om een HLA-gebaseerde detectie techniek te optimaliseren. Resultaten waren echter teleurstellend omwille van de hoge achtergrondkleuring welke niet gereduceerd kon worden ondanks verschillende optimalisatiepogingen. Er kon daarom geen software ontwikkeld worden voor de automatische detectie van deze zeldzame cellen. Optimalisatie van kleuringstechnieken en ontwikkeling van meer gesofisticeerde detectie methoden is vereist om vrouwelijke foetale microchimere cellen te visualiseren in bloed en weefsels van patiëntes met een auto-immune (schildklier)aandoening. Eenmaal deze doelstelling bereikt is, kan ook vrouwelijke microchimerie in mannen met een auto-immune aandoening bestudeerd worden.

Enkel de aanwezigheid van foetale microchimere cellen in de schildklier, waar de immuunreactie plaats vindt, werd bewezen [7-9]. Om de potentiele rol van foetale microchimere cellen in AITD op te helderen, moeten deze cellen in de schildklier gekarakteriseerd worden. Momenteel worden preliminaire data verwerkt omtrent de foetale cellen in schildklieren van patiëntes met GD. Hoewel

foetale cellen gedetecteerd werden in hun schildklieren, bevestigden de karakteriseringsstudies (nog) niet de aanwezigheid van foetale T of B cellen. Verder onderzoek om het foetale celtype in de schildklier te bepalen is noodzakelijk om een mogelijks potentieel schadelijk effect van deze cellen in auto-immune schildklieraandoeningen toe te schrijven. Indien de foetale microchimere cellen in de schildklier geen T of B cellen blijken te zijn, dan zullen verdere kleuringen geoptimaliseerd moeten worden om het foetale celtype te bepalen.

Het eerste deel van deze doctoraatsthesis wordt afgesloten met een review waarin de mogelijke hypotheses omtrent de potentiele rol van foetale microchimere cellen in auto-immune (schildklier)aandoeningen aangehaald worden (**Deel II, Hoofdstuk 5**). Foetale cellen kunnen een nadelig, voordelig of onschuldig effect hebben voor de schildklier. Op een nadelige manier kunnen foetale cellen auto-immune aandoeningen veroorzaken door een 'graft-versus-host' reactie te initiëren, of de moeder zou een 'host-versus-graft' reactie tegen deze foetale cellen kunnen starten. Ondanks het feit dat mannelijke foetale cellen enkel gekarakteriseerd werden in het bloed van een beperkt aantal patiëntes in onze studie, werd de aanwezigheid van foetale $CD8^+$ T cellen in HT en foetale $CD4^+$ T en B cellen in GD duidelijk aangetoond. Deze resultaten ondersteunen de hypothese dat foetale microchimere cellen een nadelig effect voor de schildklier kunnen hebben waarbij de foetale cellen een 'graft-versus-host' reactie in de moeder kunnen initiëren. Onze data ondersteunen de waarde en de nood voor verder onderzoek in dit onderzoeksveld. Een voordelig effect van foetale microchimere cellen waarbij deze cellen hulp zouden kunnen bieden in het herstel van weefsels, werd voornamelijk beschreven in borst- en baarmoederhalskanker. Een derde hypothese veronderstelt dat de microchimere cellen onschuldige omstanders zijn in het proces van auto-immuniteit aangezien microchimere cellen ook gedetecteerd werden in gezonde vrouwen. In onze opinie echter, hangt het nadelig, voordelig of onschuldig effect van foetale microchimere cellen voornamelijk af van een aantal factoren zoals het foetale celtype dat verkregen werd, de weefselomgeving en het type van aandoening. Nog belangrijker blijkt dat HLA-gelijkenissen tussen foetale en maternale cellen het potentieel hebben om de balans tussen voordelige en nadelige gevolgen voor de moeder te beïnvloeden. Hoe meer foetale microchimere cellen HLA-gelijkenissen vertonen met maternale cellen, hoe minder waarschijnlijk zij herkend worden door het maternale immuunsysteem en hoe meer waarschijnlijk zij het potentieel hebben om een 'graft-versus-host' reactie te starten eenmaal zij geactiveerd worden door nog onbekende mechanismen. Verder onderzoek om deze hypothese te bevestigen is noodzakelijk.

Inzicht in het mechanisme hoe foetale microchimere cellen een potentieel schadelijk effect kunnen hebben in het ontstaan van auto-immune schildklieraandoeningen, zou aanleiding kunnen geven tot

de ontwikkeling van een specifieke therapie die de foetale immuun cellen als target heeft. In de ziekte van Graves bijvoorbeeld, zouden foetale B cellen, die mogelijks verantwoordelijk zijn voor de productie van anti-schildklier stimulerende antilichamen die leiden tot hyperthyroïdie, geëlimineerd kunnen worden door Rituximab, een monoklonaal antilichaam tegen CD20, een oppervlakte merker van B cellen. Rituximab wordt reeds gebruikt in de behandeling van de auto-immune aandoening reumatoïde artritis [10]. Een bijwerking van deze depletietherapie echter is het feit dat ook niet-autoreactieve B cellen worden gedepleteerd. Daarom is het noodzakelijk om op zoek te gaan naar een therapie die specifiek de foetale cellen als target heeft. De eerste stap daarin echter, is inzicht te verkrijgen in het mechanisme hoe foetale microchimere cellen een auto-immune reactie in de moeder zouden kunnen starten.

In een tweede deel van deze thesis werden twee visualisatietechnieken geoptimaliseerd voor hun gebruik in forensische toepassingen (**Deel III**). In ons labo werden verschillende (fluorescente) kleuringen ontwikkeld voor de analyse van forensische stalen. In geval van seksuele aanranding bijvoorbeeld, kan FISH gebruikt worden om mannelijke cellen van de aanvaller te onderscheiden van vrouwelijke cellen van het slachtoffer. Vervolgens kunnen mannelijke cellen geïsoleerd worden door middel van laser microdissectie om een enkelvoudig DNA profiel van de dader te verkrijgen in plaats van een DNA-mengprofiel wat moeilijker te interpreteren is [2].

De fluorescente kleurstof DAPI heeft ook zijn toepassing in forensisch onderzoek. Het gebruik van DAPI werd geëvalueerd om nucleair DNA in haarwortels aan te kleuren om het DNA analyse slaagpercentage van het haar te kunnen voorspellen (**Deel III, Hoofdstuk 2**). Humane haren worden frequent als forensisch bewijs op misdaadplaatsen verzameld aangezien mensen ongeveer 150 haren per dag verliezen. Deze haren kunnen microscopisch vergeleken worden met referentie haren van de vermoedelijke dader. Het onderscheidingsvermogen van microscopisch haaronderzoek is echter relatief klein. ‘Short Tandem Repeat’ (STR) analyse van de haren zou daarom uitgevoerd moeten worden om de donor van het haar te identificeren. Het slaagpercentage van STR analyse van haren is echter zeer laag en negatieve resultaten van haaranalyse worden frequent gerapporteerd. Om het slaagpercentage te doen stijgen, zou een screeningsmethode voorafgaand aan de DNA analyse uitgevoerd moeten worden om de haren met nucleair DNA te selecteren. Nucleair DNA in haarwortels kan gekleurd worden met haematoxyline [11]. Haematoxyline vermindert echter de DNA opbrengst en is daarom niet aanbevolen als de haarwortels na kleuring nog moeten gebruikt worden voor DNA analyse. Een andere methode om nucleair DNA te visualiseren, is door het nucleair DNA *in situ* te labelen [12]. Zoals eerder vermeld is *in situ* labelen een zeer arbeidsintensieve techniek. Daarom werd voorgesteld om nucleair DNA in haarwortels te kleuren met DAPI met een incubatie

overnacht [13]. De huidige Belgische DNA wet eist een forensisch DNA rapport binnen de maand. Daarom moeten protocollen zo kort mogelijk gehouden worden. Het doel van deze studie was om de kleuringstechniek te optimaliseren en in te korten om te kunnen gebruiken in forensische DNA laboratoria. We waren in staat om de incubatie tijd te reduceren van een kleuring overnacht tot een directe kleuring en visualisatie van de haarwortel onder de fluorescentiemicroscoop. DNA analyse van de haarwortels waarbij kernen werden geobserveerd, resulteerde in een slaagpercentage van 94%. Zonder deze screeningsmethode zou echter maar een slaagpercentage van 30% bereikt worden, wat de waarde van onze screeningsmethode voorafgaand aan DNA analyse aantoont. Indien haren verzameld worden van kleeffilms, kan het interessant zijn voor DNA analyse om het deel van de kleeffilm te includeren daar waar de haarwortel gelokaliseerd was. Er werd namelijk een verlies van kernen gezien na verwijderen van de haarwortel van de kleeffilm. In een optimale situatie zouden haren zonder zichtbare kernen niet geselecteerd worden voor DNA analyse aangezien geen DNA profiel kon bepaald worden in 96% van de geanalyseerde stalen. In 4% van deze gevallen echter, konden volledige of gedeeltelijke DNA profielen bepaald worden. Dit zou te wijten kunnen zijn aan materiaal rond de haarwortel. Het resultaat van de DAPI kleuring zou daarom altijd beschouwd moeten worden in functie van het belang van de bewijswaarde van het gevonden haar. Als het haar het enige biologische bewijs is in een forensische zaak en de kleuring wordt negatief beschouwd, zou men kunnen overwegen om het haar toch te analyseren. Meerdere haren met dezelfde eigenschappen zouden samen genomen kunnen worden voor DNA analyse, waardoor de kans op een DNA profiel eventueel verhoogd zou kunnen worden. Indien nog steeds geen DNA profiel bepaald kan worden, zou men kunnen overwegen om een mitochondriaal DNA-profiel op het restant van het haar te bepalen. Mitochondriaal DNA is echter hetzelfde in de maternale lijn en is daarom minder onderscheidend in vergelijking met autosomale STR analyse.

Deze snelle screeningsmethode is momenteel geïmplementeerd in ons forensisch DNA laboratorium. Gelijkaardige resultaten werden verkregen op haarwortels die geselecteerd werden uit 36 forensische zaken. Zonder deze screeningsmethode zou men 279 haarwortels geanalyseerd hebben, wat gepaard gaat met een hoge gerechtskost. Op basis van de aanwezigheid van kernen in de haarwortel, werden uiteindelijk maar 16 haren geselecteerd voor DNA analyse. Er werd een DNA profiel verkregen in 81% van de gevallen. In de resterende 19% van de haarwortels echter, werden minder dan 20 kernen geobserveerd. Uit experimentele data bleek echter dat minimum 20 nuclei noodzakelijk zijn voor op zijn minst gedeeltelijke DNA profielen te kunnen verkrijgen. Bovendien moeten we rekening houden met het feit dat ongunstige weersomstandigheden voorafgaand aan het verzamelen van de haren op de plaats van de misdaad, een invloed kunnen hebben op de resultaten. Deze data tonen de waarde van deze screeningsmethode aan om de gerechtskosten te beperken tot

een minimum. Wij bevelen alle forensische DNA laboratoria aan om deze snelle screeningsmethode toe te passen voor het selecteren van haren die geschikt zijn voor DNA analyse.

Een van de eerste stappen in het analyseren van forensisch bewijs, is de zoektocht naar biologisch materiaal dat kan gebruikt worden voor DNA extractie en analyse. Bloed is een vaak voorkomend lichaamsvloeistof dat gedetecteerd wordt op overtuigingsstukken gevonden op de plaats van de misdaad, vooral indien het gaat om misdaden met geweld. Bloedvlekken op donkere achtergronden echter, bijvoorbeeld op donkere stoffen, zijn moeilijk zichtbaar met het blote oog. Verschillende visualisatietechnieken zoals Polilight, infrarood detectie, luminol gebaseerde detectietechnieken en fluoresceïne, werden voorgesteld om bloedvlekken op donkere achtergronden te lokaliseren. Er zijn echter één of meerdere nadelen verbonden met deze technieken, zoals de nood om te werken in een donkere kamer, de nood aan alternatieve lichtbronnen, het voorkomen van vals positieve of vals negatieve resultaten, een hoge gerechtskost of een negatief effect op DNA. Het doel van deze studie was om een eenvoudige, goedkope visualisatietechniek te ontwikkelen zonder de nood aan een alternatieve lichtbron (**Deel III, Hoofdstuk 3**). De voorgestelde visualisatietest is gebaseerd op de transfer van bloed naar een filterpapier doorheen capillaire krachten om bloedvlekken op een donker stuk stof te kunnen lokaliseren. Kleine hoeveelheden onverdund bloed op verschillende soorten stoffen kunnen gemakkelijk gedetecteerd worden met deze techniek. Dit is uitermate belangrijk aangezien de meeste bloedvlekken op forensisch bewijs onverdund en in minieme hoeveelheden aanwezig zijn. In geval van verdunde bloedvlekken worden enigszins betere resultaten verkregen op los geweven, natuurlijke stoffen in vergelijking met vast geweven, synthetische stoffen. Aangezien deze visualisatietechniek enkel een hulpmiddel is om bloedvlekken te visualiseren op donkere stoffen, zou een positief resultaat altijd bevestigd moeten worden met een algemene bloedtest, zoals de Kastle-Meyer test.

Deze algemene bloedtest kan dan uitgevoerd worden op de gedetecteerde bloedvlek op de stof, maar het kan ook rechtstreeks uitgevoerd worden op het filterpapier van de visualisatietest, wat een belangrijk voordeel is van de voorgestelde techniek. De humane oorsprong van de bloedvlek kan bevestigd worden met een Hexagon OBTI test. Indien geen bloedvlekken worden gevisualiseerd op het filterpapier, dan kan dit betekenen dat er ofwel geen bloed aanwezig is op de stof of dat het bloed meer verdund is dan wat visueel gezien wordt met de test. In dat geval kan men overwegen om meer sensitieve en duurdere bloedtesten uit te voeren op verschillende locaties op de stof. Echter, de belangrijkste bloedvlekken die bruikbaar zijn voor DNA analyse zullen gedetecteerd worden met deze visualisatietechniek. Naast de lage kost en het gemak om deze test uit te voeren, 2 belangrijke voordelen in een routine forensisch DNA laboratorium, kan DNA analyse ook direct

uitgevoerd worden op de getransfereerde bloedvlek op het filterpapier zonder dat de bloedvlek uitgeknipt dient te worden uit het bewijsstuk. Indien geen DNA profiel verkregen kan worden van de bloedvlek op het filterpapier, dient de vlek op de stof zelf uitgeknipt te worden voor DNA analyse.

De visualisatietechniek voorafgaand aan andere bloedtesten om latente bloedvlekken op donkere stoffen op te sporen, is ten zeerste aanbevolen aan forensische laboratoria aangezien het zeer goedkoop en makkelijk uit te voeren is. Bovendien kan het patroon van de bloedvlek, wat een belangrijk onderdeel is van de misdaadreconstructie, uitgevoerd worden op het filterpapier zelf. Van nu af aan zal deze visualisatietechniek gebruikt worden in ons forensisch DNA laboratorium om bloedvlekken op donkere stoffen zichtbaar te maken. Meer onderzoek op mengsels van bloed en andere biologische substanties zoals speeksel en braaksel, semen en menstrueel bloed, is echter noodzakelijk om de invloed van deze substanties op de visualisatie na te gaan, alsook de invloed van deze substanties op de daaropvolgende DNA analyse uitgevoerd op de vlek op het filter papier.

Gedurende deze PhD thesis werden verschillende visualisatietechnieken geoptimaliseerd en gevalideerd om klinische en forensische stalen te analyseren. De continue ontwikkeling van nieuwe visualisatietechnieken is echter noodzakelijk om gemakkelijker deze stalen te analyseren.

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ADDENDUM

Male fetal microchimeric cells in blood and thyroid glands of women with an autoimmune thyroid disease

Invited addendum

Trees Lepez, Mado Vandewoestyne, Dieter Deforce

Adapted from Chimerism 2012; 3(1), 1-3

Abstract

Persistence of fetal microchimeric cells may result in the development of autoimmune thyroid diseases (AITDs) such as Hashimoto's thyroiditis (HT) or Graves' disease (GD). In women, HT and GD show an increased incidence in the years following parturition. Although fetal cells have already been shown to be more common in the thyroid glands of patients with an AITD compared to controls, these cells haven't been described in blood of these patients. Our study detected male fetal cells in blood of all patients with an AITD. Moreover, male fetal cells were immune cells potentially capable of initiating a graft-versus-host reaction and suggest a potential role of these cells in the pathogenesis of AITD. Our study indicates the value and need for further research in this field.

During pregnancy, fetal cells cross the placenta into the maternal circulation [1,2] and can persist in the postpartum period in tissues such as the thyroid gland [3]. The mother becomes microchimeric [4-6]. The persistence of these fetal cells may result in the development of autoimmune thyroid diseases (AITD) [7,8] such as Hashimoto's thyroiditis (HT) and Graves' disease (GD) [9-11].

The presence of male fetal cells can be investigated by real-time PCR or Fluorescence *In* Situ Hybridization (FISH) using male-specific markers in women who had given birth to a son. While real-time PCR only indicates the presence of fetal cells and estimates the amount of fetal cells [7], FISH gives an exact number [5,6]. Therefore, data obtained by real-time PCR are hard to compare with those obtained by FISH, as shown by Renne *et al.* [5]. Using real-time PCR, the authors detected male fetal microchimerism in 38% of the patients with HT, compared to 83% using FISH. The differences might be explained by different sensitivities of both techniques [12]. With real-time PCR, a single male cell can be detected within a background of 100.000 female cells [13] compared to 1 male cell within 2.000.000 female cells with FISH [14]. Therefore, our study used the latter technique to detect male fetal cells in blood of women with an AITD.

Male fetal microchimerism has already been shown to be more common in the thyroid glands of patients with AITD compared to controls [4-6,15,16]. Using real-time PCR, Klintshar *et al.* detected male fetal cells in 47% of the thyroid glands of patients with AITD compared to 4% of women with nodular goiter [4]. Later, the authors expanded the inquiry with a quantitative PCR-based approach, amplifying the *DYS14* region of the Y chromosome, a technique that allows greater sensitivity because of multiple repeats [15]. This study identified male DNA in 38% of women with HT, in 5% of women with multinodular goiter and 0% of women with normal thyroid glands. In 20% of patients with GD, male fetal cells were detected in paraffin-embedded thyroid tissue compared to 0% in women with adenoma [16]. However, examining fresh-frozen thyroid tissues, male fetal microchimeric cells were detected in 85% of patients with GD compared to 25% of patients with adenoma, showing that paraffin-embedded tissue is subject to DNA fragmentation. Using FISH, Renne *et al.* [5] found that 60% of women with HT, 40% of women with GD and 22% of patients with thyroid adenoma were positive for male fetal cells in the thyroid. Using the same technique, Srivatsa *et al.* [6] detected male fetal cells in 72% of women with an AITD compared to 0% in healthy controls.

In contrast to thyroid tissue, male fetal cells were detected in blood of all patients with an AITD in our study [14]. The highest number of male fetal cells was observed in patients with GD (14 to 29 male fetal cells per million maternal cells), followed by HT (7 to 11) compared to the low number of male fetal cells detected in healthy volunteers (0 to 5). This indicates a higher degree of

microchimerism in AITD compared to healthy controls ($p < 0.05$). Moreover, significantly more male fetal cells were detected in patients with GD compared to patients with HT ($p = 0.0061$) [14].

The etiologic consequences of fetal microchimerism are difficult to assess to date. Up to now, only the presence of fetal engrafted cells in AITD is proven, but not an actual active role of microchimerism in the autoimmune process. An argument against an active role is that only a part of all patients with AITD show male microchimerism in their thyroid [4-6,15,16]. Nevertheless, in patients who appear to be negative, it is possible that male fetal microchimerism is not detectable by the methods used or the fact that only female fetal cells are present [4]. In our study however, male fetal cells were detected in all patients with an AITD. Taken together, these data suggest a potential role of these cells in the pathogenesis of AITD [13,17].

If fetal cells indeed play a role in AITD, it is expected that fetal cells are pluripotent stem cells or immune cells. Male fetal $CD34^+$ and $CD34^+/CD38^+$ progenitor cells, capable of differentiating into immune competent cells [3], but also mature male fetal T, B and NK cells [18] have been isolated from the blood of women with scleroderma, an autoimmune disease of the skin. Cha *et al.* [19] suggested that fetal progenitor cells may differentiate in the maternal host and might alter immune function. Fetal immune cells may be reactive to maternal antigens [20] and, therefore, have the capacity to trigger graft-versus-host reactions. It is possible that fetal cells also elicit an intrathyroidal graft-versus-host reaction that leads to AITD [5]. Ando *et al.* [16] and Davies *et al.* [21] propose that after delivery, when placental tolerogenic mechanisms are lost, intrathyroidal fetal immune cells are activated and initiate a graft-versus-host reaction against maternal antigens resulting in the activation of maternal autoreactive T cells which could eventually modulate AITD postpartum.

Our study focused on the presence of male fetal B and T cells in blood of women with an AITD because these subsets are more likely to initiate or be involved in immune response. Mainly male fetal $CD8^+$ cytotoxic T cells were found in blood of patients with HT, possibly causing cell death leading to hypothyroidism [14,22]. In blood of patients with GD, mainly male fetal B cells were found which are possibly activated by male fetal $CD4^+$ T cells, also detected in their blood [14]. Activated B cells could secrete TSHR-stimulating antibodies which could lead to hyperthyroidism [22]. Other male fetal cell types not isolated during selection of T and B cells, are likely to be natural killer (NK) cells or hematopoietic progenitor cells capable of differentiating into immune competent cells [18]. Our study indicates the value and need for further research in this field.

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Curriculum Vitae

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2000 – 2002:	Sciences- Mathematics; Sint-Bernardus College Oudenaarde
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2006 – 2008:	Master in Forensics, Criminology and Law, Maastricht University Thesis: Wie is het? DNA-onderzoek in strafzaken in verband met het vaststellen van uiterlijk waarneembare persoonskenmerken
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Research fellowship

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Bibliography

A1 publications

- Fetal microchimeric cells in blood of women with an autoimmune thyroid disease
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  PLoS ONE 2011; 6(12): e29646
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  Mado Vandewoestyne, Björn Heindryckx, Trees Lepez, Rudy Van Coster, Jan Gerris, Petra De Sutter, Dieter Deforce
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  Mitochondrion 2012; 12(4): 477-479.
- A novel dictionary based computer vision method for the detection of cell nuclei
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- Fast nuclear staining of hair roots as a screening method for successful STR analysis in forensics
  Trees Lepez*, Mado Vandewoestyne*, David Van Hoofstat, Dieter Deforce (* equal contribution)
  Submitted to Forensic Science International: Genetics
- Evaluation of a visualization assay for blood on forensic evidence
  Mado Vandewoestyne*, Trees Lepez*, David Van Hoofstat, Dieter Deforce (* equal contribution)
  Accepted for publication in Journal of Forensic Sciences
- Detailed method description for non-invasive monitoring of differentiation status of human embryonic stem cells
  Ellen Scheerlinck, Katleen Van Steendam, Mado Vandewoestyne, Trees Lepez, Veerle Gobin, Paulien Meert, Liesbeth Vossaert, Filip Van Nieuwerburgh, Ann Van Soom, Luc Peelman, Björn Heindryckx, Petra De Sutter, Maarten Dhaenens, Dieter Deforce
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A2 publications

- Fetal microchimeric cells in blood and thyroid glands of women with an autoimmune thyroid disease
  Trees Lepez, Mado Vandewoestyne, Dieter Deforce
  Chimerism 2012; 3(1): 21-23
- Fetal microchimeric cells in autoimmune thyroid diseases: harmful, beneficial or innocent for the thyroid gland?
  Trees Lepez, Mado Vandewoestyne, Dieter Deforce
  Chimerism 2013; 4(3): 1-8

Oral and poster presentations

- Oral presentation at the conference on "Laser microdissection: a day for answers" (BioPark, Herfordshire, Welwyn Garden City, UK (17/06/2010))
  Laser microdissection in forensic sciences
  Trees Lepez, Mado Vandewoestyne, David Van Hoofstat, Dieter Deforce
- Poster presentation at the 8th international autoimmunity congress (Granada, Spain (9-13/05/2012)):
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Educational experience

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